Receptor Recognition and Response of Dendritic Cells to Biomaterials

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Receptor Recognition and Response of Dendritic Cells to Biomaterials

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SUMMARY

Tissue engineered devices aim to emerge as feasible alternatives to organ or tissue transplants, which are very limited in availability. As combination products, they are composed of both a biomaterial (scaffold) and biological component (cells, proteins, etc). Upon implantation into the body, the host response to this construct will be a combination of both a non-specific inflammatory response to the biomaterial and an antigen-specific immune response toward an allo- or xenogeneic biologic (Babensee et al. 1998). Recently, it has been shown that the response to the biomaterial component may influence the immune response to the co-delivered cellular component. More specifically, there is evidence of a biomaterial adjuvant effect as poly(lactic-*co*-glycolic) acid (PLGA) scaffolds or microparticles increase an immune response to a model antigen, ovalbumin (Matzelle and Babensee 2004; Bennewitz and Babensee 2005; Norton et al. 2010).

It is hypothesized that dendritic cells (DCs), the major antigen-presenting cells of the body, play an important role in this response as they serve to link the inflammatory response to an adaptive immune response. Activation (or maturation) of DCs in the presence of the biomaterial-delivered biologic component may facilitate increased humoral immunity. In support of this, PLGA film or microparticle (MP) treatment alone is capable of inducing DC maturation while a biomaterial film, which does not support an adjuvant effect (e.g. agarose) (Norton et al. 2010) does not support DC maturation *in vitro* (Yoshida and Babensee 2004; Yoshida and Babensee 2006; Yoshida and Babensee 2006). Therefore, elucidating how DCs recognize and respond to biomaterials may be a critical factor in understanding how biomaterials modulate immune responses.

The mechanism of DC recognition and response to biomaterials is unknown, and the major intended goal of the research presented herein was to further elucidate the manner of biomaterial-induced DC maturation. It was hypothesized that the host response to a biomaterial implant may be mediated by 'danger signals' (endogenous molecules), which are capable of eliciting inflammation and simultaneously acting as adjuvants. To examine the potential role of 'danger signals' in the host response to a biomaterial, polyethylene terephthalate (PET) discs were implanted for 16hr in the peritoneal cavity (as a measurement of acute inflammation) or for 2 weeks subcutaneously (as a measurement of chronic inflammation) in wild-type or toll-like-receptor 4 (TLR4)-deficient mice (CHAPTER 4). Following 16hr implantation, there was no difference in either total or differential lavage leukocyte profiles; however, there was significant delayed inflammatory response in the TLR4-deficient mice as seen in the altered biomaterial-adherent leukocyte profile (CHAPTER 4). TLR4-deficient mice show a significant reduction in monocyte/macrophage presence as well as a significant increase in neutrophil presence on the biomaterial surface, as compared to wild-type controls. However, both strains exhibited similar pro-inflammatory cytokine production (TNF-α) in the peritoneal cavity in response to the biomaterial implant in a TLR4-independent manner (CHAPTER 4). Following 2 weeks subcutaneous implantation, both strains induced similar fibrous capsule thickness implying that there were compensatory mechanisms during the chronic inflammatory response (CHAPTER 4). Therefore, it appeared that TLR4 / 'danger signal' interactions may have only contributed to the host response to a biomaterial during an immediate to early time frame during which they aid in directing the adhesive leukocyte profile.

To isolate the role of biomaterial-induced TLR4 signaling in the form of nuclear factor (NF)-κB and activator protein (AP)-1 transcription factor activation, TLR4-expressing human embryonic kidney (HEK) 293 cells were used, as were MyD88-

silenced transformed TLR4-expressing HEK293 cells and wild-type HEK293 cells for negative controls (CHAPTER 5). TLR4-expressing HEK293 cells were found to respond to LPS treatment as seen in increases in activated NF-κB and AP-1 family members in nuclear extracts as well as induction of IL-8 secretion (CHAPTER 5). Interestingly, PLGA and agarose films only induced significant activation of AP-1 (but not NF-κB) family members and while not inducing IL-8 production (CHAPTER 5). To assure the role of TLR4, TLR4-expressing HEK293 cells were stably silenced for MyD88 expression to inhibit TLR4-induced signaling. However, MyD88-silenced TLR4-expressing HEK293 cells were still able to respond to LPS as seen in activation of NF-κB (CHAPTER 5). This may be due to LPS/TLR4 signaling through a known MyD88-independent pathway. Regardless, PLGA or agarose treated wild-type HEK293 cells, which have been shown to express little to no TLR4, were still found to have significant induction of AP-1 activation (CHAPTER 5). Therefore, though HEK293 cells seemed to respond to biomaterials through AP-1, this appeared to be in a TLR4-independent manner.

Upon examination into the role of TLR4 in the response of DCs to biomaterials, bone marrow derived DCs (BMDC) from $TLR4^+$ mice were found to show signs of maturation (increase in CD86 expression) when treated with PLGA films, which was not found in BMDCs from $TLR4^-$ mice (CHAPTER 5). Also, PLGA films and MPs induced significant signs of early apoptosis in a TLR4-dependent manner. However, neither PLGA films nor MPs induced significant amounts of TNF-α secretion from $TLR4^+$ BMDCs as was found when stimulated with ultrapure-LPS (CHAPTER 5). This may have been due, in part, to BMDC pre-activation as was detected in their expression of high levels of major histocompatibility complex (MHC) class II molecules and CD80/CD86, prior to biomaterial treatment (CHAPTER 6). Upon further investigation, it was found that during BMDC development, adherent BMDCs are substantially more responsive to

maturation-inducing stimuli than non/loosely adherent BMDCs, which had been previously only been investigated (CHAPTER 6). This was found in BMDCs across three separate strains of mice (C57BL/6, C57BL/10 and C57BL/10ScSn) in response to ultrapure-LPS, PLGA films or PLGA MPs (CHAPTER 6). Therefore, the role of TLR4 in biomaterial-induced DC maturation was further examined analyzing the response of both adherent and non/loosely adherent BMDC fractions to PLGA film or MP treatment (CHAPTER 6). Adherent BMDCs from $TLR4^{+}$ mice were more responsive to both ultrapure-LPS and PLGA film treatment as anticipated; however, adherent BMDCs from $TLR4^{-}$ mice still showed signs of biomaterial-induced DC maturation when treated with PLGA films (CHAPTER 6). Therefore, the role of TLR4 in DC-biomaterial recognition was inconclusive using this particular TLR4-deficient strain and appropriate control strains.

To examine the potential role of other TLRs, gene expression of all TLRs following biomaterial treatment was investigated in human monocyte-derived DCs. It was anticipated that TLRs involved in the response to biomaterial would be down regulated following treatment. However, ultrapure-LPS (a TLR4 specific ligand) induced down-regulation of several TLRs (including TLR4) indicating that it may not be possible to further deduce specific TLR involvement in biomaterial recognition even if biomaterial treatment resulted in down-regulation of a TLR family member (CHAPTER 5). However, TLR-related gene expression patterns induced by PLGA were found to loosely match that of ultrapure-LPS treatment while patterns induced by agarose closely matched that of untreated controls. This further verified PLGA as a stimulatory biomaterial, which induced overall gene expression patterns similar to that of a bacterial by-product. Agarose treatment influenced overall gene expression minimally and implied that agarose may maintain DCs in an immature phenotype (CHAPTER 5). Differential transcription factor up-regulation by LPS (NF-κB), PLGA (Elk-1) or agarose (cFos/AP-1)

was attempted to be validated via western blotting. However, only NF-κB, and not Elk-1 nor cFos, was found to match increased RNA expression with increased total or activated protein expression (CHAPTER 5).

Dendritic cells were found to readily adhere to the activating biomaterial PLGA but not to tissue culture polystyrene (CHAPTER 7). Therefore, the role of adhesion to biomaterial-induced DC maturation was investigated. Using a human monocyte-derived DC culture, DCs were found to preferentially adhere to PLGA, while other contaminating cells (e.g. B cells) were not found to adhere as determined via immunofluorescence (CHAPTER 7). Human DCs cultured on PLGA (but not agarose) showed increases in integrin expression across both β_1 and β_2 family members (CHAPTER 7). However, using antibody-blocking methods, anti-β_2 (but not anti-β_1) treatments inhibited adhesion to biomaterials (CHAPTER 7). Furthermore, β_2 integrin mediated DC adhesion was found to play a significant role in controlling levels of DC maturation when cultured on biomaterials (CHAPTER 7). The significant decreases in the level of DC adhesion during anti-β_2 treatment resulted in significantly higher presence of immature DCs (lower CD86 expression) (CHAPTER 7). Dendritic cells also showed significant presence of β_2 integrins in podosomes that directly interacted with biomaterial surfaces, which was not found with β_1 integrins (CHAPTER 7).

Overall, these findings illustrate that an adhesive biomaterial such as PLGA may induce maturation in a β_2 integrin adhesion dependent manner, which may potentially allow for other stimulatory receptor (e.g. TLR4) engagement. Using hydrogel biomaterials such as agarose for tissue-engineered devices, which may inhibit protein adsorption and subsequent cellular adhesion due hydrophilic properties, may prevent β_2 mediated adhesion and potentially minimize biomaterial-induced DC maturation.

CHAPTER 1

INTRODUCTION

Upon introduction to the body, biomaterials induce a concerted host response stemming from the recognition of the implant as a foreign object. This response often limits the efficacy of the material to fulfill its intended therapeutic application due to a non-specific inflammatory. Biomaterials have been employed as scaffolds for tissue-engineering devices as well as for vaccine delivery vehicles. Tissue-engineered devices, which combine biological and biomaterial components, aim to alleviate the dramatic shortage of transplants needed for organ and tissue loss/failure (Langer and Vacanti 1993). For these combination products, there is an inherent need to not only minimize the non-specific inflammatory response to the biomaterial component but also reduce the antigen-specific immune response directed toward the biologic to assure therapeutic efficacy (Babensee et al. 1998). The immune response is particularly relevant during the delivery of allo- or xenogeneic biological components, which are likely candidates or therapy in order to assure “off-the-shelf” availability of devices (Nerem 2006). This is in contrast to biomaterial-delivered vaccines which seek to maximize a humoral immune response toward the biologic (Babensee 2008).

Previously, it has been shown that a biomaterial may contribute to directing the immune response toward an antigen. While certain biomaterials act as an adjuvant (e.g. PLGA), others actually minimize the immune response to a co-delivered protein (e.g. agarose) (Matzelle and Babensee 2004; Bennewitz and Babensee 2005; Norton et al. 2010). Subsequently, it was hypothesized that DCs may provide an explanation, in part, for the adjuvant effect of biomaterials. Human DCs mature upon contact with tissue-engineering relevant biomaterials such as chitosan, PLGA and alginate while other materials such as hyaluronic acid (HA) or agarose minimize or potentially inhibit DC

maturation *in vitro* (Yoshida and Babensee 2004; Babensee and Paranjpe 2005; Yoshida and Babensee 2006). It is believed that this biomaterial-manipulation of DC phenotype *in vivo* may control the adaptive immune response to a co-delivered antigen due to increased or decreased T cell stimulatory capacity of DCs which influence T cell proliferation with subsequent effects of increased antibody production by B cells. Currently, the mechanism by which DCs recognize and respond to biomaterials, as with many biomaterial-mediated cellular responses, is unknown.

The objective of this research was to further understand how DCs recognize and respond to biomaterials. The *central hypothesis* was that biomaterial-adsorbed proteins facilitate the recognition of a biomaterial as a foreign object, and these molecules propagate the activation of cells both *in vitro* and *in vivo* through direct receptor recognition. More specifically, there was an interest in determining the role of 'danger signals' in this recognition, which are molecules capable of directly stimulating host and DC responses. After recognizing the biomaterial as a foreign object via the adsorbed protein layer, DCs may respond through signaling pathways to induce increased gene expression of molecules associated with DC maturation such as co-stimulatory, major histocompatibility molecules and cytokines. It was hypothesized that an activating biomaterial surface (such as PLGA) may present or act as a depot for stimulatory 'danger signal' molecules from serum (*in vitro*) or from host blood/tissues (*in vivo*, following implantation) that elicits the recognition and response of a biomaterial as a foreign object, particularly by DCs.

Specific Aim 1: Determine the relevance of 'danger signals' on biomaterial surfaces from the DC response to biomaterials through TLR4 *in vitro* and the role of TLR4 to participate in the acute and chronic inflammatory response to a biomaterial implant. The *working hypothesis* for this aim was that cells recognize and

respond to adsorbed ‘danger signals’ on the surface of biomaterials. Particularly, the role of Toll-like Receptor (TLR) 4 was hypothesized to play a role in this process because of its known recognition of many endogenous molecules and contribution to the host response following tissue damage. The role of TLR4 in biomaterial-induced DC maturation was investigated using bone marrow-derived DCs (BMDCs) from TLR4-deficient mice. Dendritic cells were cultured with biomaterial PLGA films or microparticles (MP). Non/loosely adherent and adherent BMDCs were examined for maturation marker expression for biomaterial treatment by flow cytometry and pro-inflammatory cytokine secretion and compared to that of DCs from two appropriate TLR4-wild type control mice. TLR4-induced biomaterial signaling was isolated away from DCs through the use of three model cell lines cultured on biomaterial (PLGA or agarose) films: 1. TLR4-expressing HEK293 cells, 2. TLR4-expressing HEK293 cells, which have been stably silenced for MyD88; 3. Wild-type HEK293 cells which express little to no surface TLR4. NF-κB and AP-1 transcription factor activation was examined in nuclear extracts, interleukin (IL)-8 secretion was characterized and apoptosis markers caspase 8 and 3 were also analyzed. The gene expression of TLRs and TLR-related molecules was also investigated in PLGA or agarose treated human DCs to attempt to determine other potential TLRs involved in the biomaterial recognition process.

Lastly, the most relevant context to study ‘danger signals’ role in the response to a biomaterial is an *in vivo* setting where implantation-induced damage causes release of intracellular molecules and extracellular tissue fragments. These ‘danger signals’ may induce activation of various cell types through TLR4 (or other TLRs). The role of TLR4 in the acute and chronic host response to a biomaterial implant was studied using a model biomaterial (polyethylene terephthalate, PET) implanted intraperitoneally (i.p.) or subcutaneously (s.c.) in TLR4-deficient mice. Leukocyte recruitment, differential cell counts and cytokine presence was assessed following 16 hr (i.p.) along with fibrous

encapsulation following 2-week implantation (s.c.) and compared to that of naïve and sham surgery controls.

Specific Aim 2: Determine the role of integrin-mediated adhesion in biomaterial-induced DC maturation. The *working hypothesis* for this aim was that DC maturation caused by biomaterials is, in part, directed by integrin-mediated adhesion. Integrins family member gene expression was investigated in PLGA or agarose treated human DCs to determine putative integrins involved in biomaterial adhesion. Antibody-blocking studies were utilized to determine the functional role of β_1 and β_2 integrin family members in DC/biomaterial interaction. The role of integrins was investigated in both DC adhesion to biomaterials as well as biomaterial-induced DC maturation via cell counting and flow cytometry for maturation marker expression, respectively. Lastly, cross-linking DCs to the biomaterial surface and performing immunofluorescence for β integrin family members was used to probe the direct binding of DC-integrins.

CHAPTER 2

RESEARCH SIGNIFICANCE

Tissue-engineered devices aim to alleviate the dramatic shortage of available organ transplants by providing an engineered source of tissues using cells/biologics in combination with material scaffolds (Langer and Vacanti 1993; Stock and Vacanti 2001; Griffith and Naughton 2002; Vacanti 2006). Biomaterials for tissue-engineering, either naturally or synthetically-derived, serve to provide a directional substrate for cell adhesion and host integration upon engraftment to aid in replacing or repairing lost or damaged tissues (Stock and Vacanti 2001). However, it is essential that such combination products elicit an appropriate recipient host response, which will be a mixture of a non-specific inflammatory response toward the biomaterial component and a potential adaptive immune response toward an immunogenic cellular component (Babensee et al. 1998). It is believed that the host inflammatory response to the biomaterial may manipulate the immune response (Babensee 2008); therefore, material selection for use in tissue-engineered devices is likely critical for the success of the therapeutic biologic.

In this context, biomaterials commonly used in tissue-engineered combination products (PLGA) have been shown to act as adjuvants while others such as agarose maintain minimal immune response to a co-delivered model antigen (Matzelle and Babensee 2004; Norton et al. 2010), and even the physical form of the biomaterial-carrier can alter the response (Bennewitz and Babensee 2005). It has been hypothesized that biomaterials induce activation (or maturation) of antigen-presenting cells (APC) such as DCs *in vivo* during the innate/non-specific inflammatory host response to the material. Since DCs link innate and adaptive immunity, the two major branches of the immune system, it is believed that biomaterial-induced DC maturation *in*

vivo during the host response may lead to increased T cell presentation/activation toward any associated foreign antigen. In support of this, adjuvant-inducing biomaterials (e.g. PLGA, chitosan) have been found to induce DC maturation and subsequent increase in T cell activation *in vitro* while non-adjuvant biomaterials (e.g agarose) do not (Yoshida and Babensee 2004; Babensee and Paranjpe 2005; Yoshida and Babensee 2006; Yoshida et al. 2007). Understanding this differential DC response across materials may be critical to elucidating how a biomaterial-component may modulate an immune response to a combination product. This would further strengthen the criteria for material selection for tissue-engineered devices, which seek to minimize a biomaterial adjuvant effect and also contribute to the growing field of biomaterial vaccine vehicles, which seek to maximize this immune response.

The aim of this work was to improve understanding of how DCs recognize and respond to biomaterials. The major goal is to elucidate families of receptors that involved in mediating DC responses to biomaterials. With the knowledge of cellular recognition in hand, biomaterials may be designed or chosen based on the specific application for the combination product. For tissue-engineered constructs, a biomaterial scaffold could be selected which limits the recognition by these critical DC receptors with the aim of minimizing an adaptive immune response to co-delivered cellular components. In contrast, vaccine delivery vehicles could be designed to engage these receptors to ensure an optimal humoral immune response to the distributed biologic.

CHAPTER 3

LITERATURE REVIEW

3.1. Innate and Adaptive Immunity

Innate immunity involves the sentinel cells of the immune system that are initial responders upon microbe entry into the body. Leukocytes (neutrophils, monocytes/macrophages, dendritic cells, natural killer (NK) cells, etc) respond to pathogens using PRRs which recognize pathogen-associated molecular patterns (PAMPs) common across many microorganisms (Akira et al. 2006). The effector functions of these receptors include responding to opsonization (including pathogen-bound complement or IgG), triggering phagocytosis and activating inflammatory signaling cascades (Janeway and Medzhitov 2002). PRRs present on DCs include calcium dependent (C-type) lectins (van Vliet et al. 2006), Toll-like receptors (TLRs) (Kaisho and Akira 2001; Visintin et al. 2001; Janeway and Medzhitov 2002; Kawai and Akira 2006; van Vliet et al. 2007), scavenger receptors (SRs) (Peiser et al. 2002), as well as integrins / complement receptors (CRs) (Carroll 1998) and Fc-receptors (Nimmerjahn and Ravetch 2008). If an infection (or foreign antigen presence) is long-lived, an innate immune response will yield an antigen-specific adaptive immune response leading to the production of antibodies against the infecting pathogen. The adaptive immune response involves the clonal expansion of naïve T-lymphocytes (T cells) that respond to processed antigen presented by mature APCs in association with major histocompatibility complex (MHC) molecules. Depending on how the antigen is processed, it may either be displayed on MHC class I or class II molecules which coordinates with the T cell receptor (TCR) on cytotoxic ($CD8^+$) or helper T cells ($CD4^+$), respectively (Abbas et al. 2007). Along with MHC molecules, APCs also express co-stimulatory molecules (such as CD80 and CD86), which act as secondary signals for T

cell stimulation in the lymph nodes. Upon stimulation/proliferation, T cells help activate naïve B-lymphocytes (B cells) that are specific to the particular antigen by secreting cytokines (such as IL-4) inducing B-cell expansion. B cells subsequently proliferate and mature into antibody-secreting cells known as plasma cells that produce opsonizing antibodies specific for the antigenic organism originally captured and presented by APCs. This opsonization aids the recognition process of the effector cells of the innate immune response by marking the microbe for clearance via PRRs such as Fc-receptors (Bajtay et al. 2006).

3.2. Dendritic Cells and Endogenous Adjuvants ('danger signals')

Dendritic cells are professional APCs that process foreign-entities for antigen-capture and presentation to T cells. Dendritic cells are more potent APCs compared to other cell types such as macrophages or B cells due to expressing higher levels of MHC and co-stimulatory molecules following maturation (Banchereau and Steinman 1998). They are located in specific tissues (such as skin), which are common entryways of infection (Steinman and Banchereau 2007), where they exist in an 'immature' state (Banchereau and Steinman 1998). In this state they have a high capacity for endocytosis, low surface expression of MHC and co-stimulatory molecules, high surface levels of many PRRs such as Fc-receptors (Banchereau and Steinman 1998) and macrophage mannose receptor (Figdor et al. 2002). It is believed that DCs switch from an 'immature' state to a 'mature' state after encountering molecules known as adjuvants (PAMPs or 'danger signals') that induce high levels of maturation in DCs (Abbas et al. 2007). Mature DCs display 'dendritic' processes, high levels of co-stimulatory and MHC class II molecules for increased stimulation of T cells at lymph nodes, increased cytokine production for autocrine and paracrine stimulation of T cells and other cell types, and

increased chemokine receptor expression such CCR7 (Sanchez-Sanchez et al. 2006) for homing to lymph nodes (Banchereau and Steinman 1998).

Adjuvants (such as Freund's Adjuvant in mice) are delivered in association with an antigen to increase the antigen-specific humoral immune response (for vaccine purposes) by containing TLR-binding ligands that induce DC maturation through PRRs (e.g lipopolysaccharide, LPS, triggers maturation through TLR4). Along with microbial products, host-derived stimuli have been found to trigger DC maturation (termed 'danger signals' or endogenous adjuvants).

The danger model, proposed by Polly Matzinger, suggests that a host organizes an immune response to non-self antigens only when associated with cell or tissue damage (Matzinger 1994; Matzinger 2002). For example, the model proposes that an organ transplant may be rejected, in part, because DCs are activated by the release of host molecules such as intracellular molecules normally sequestered in the cytoplasm but released due to necrosis induced by the implantation procedure. Active 'danger signals' (or damage-associated molecular patterns, DAMPs) have also come to include fragmented or damaged extracellular matrix (ECM) molecules displaying hidden antigens that are typically unseen by immune cells in an organized scaffold (Rock and Kono 2008). As such, DCs are activated by endogenous molecules from stressed/dying cells, and these molecules act as adjuvants to increase the antibody production to a model antigen *in vivo* (Gallucci et al. 1999). Since this discovery, the mechanism behind 'danger signal'-mediated DC maturation has been further investigated.

Toll-like receptors (particularly TLR4 but also TLR2) have been implicated in the recognition of many endogenous molecules (Fig. 3-1). For TLR4 alone these include: plasmin (Ward et al. 2006), fibronectin (Okamura et al. 2001), elastase (Devaney et al. 2003), high mobility group box (HMGB)1 (Yu et al. 2006), hyaluronic acid fragments (Termeer et al. 2002; Jiang et al. 2005; Taylor et al. 2007), fibrinogen (Smiley et al.

2001), heat shock protein (HSP)60 (El Mezayen et al. 2007) and heparan sulfate (Takeda et al. 2003). Interestingly, many of these molecules are not intracellular (as is HSP60 and HMGB1) but are ubiquitous plasma proteins (fibrinogen, fibronectin, plasmin) that may serve to augment the inflammatory response toward infection or injury through TLR4 (El Mezayen et al. 2007).

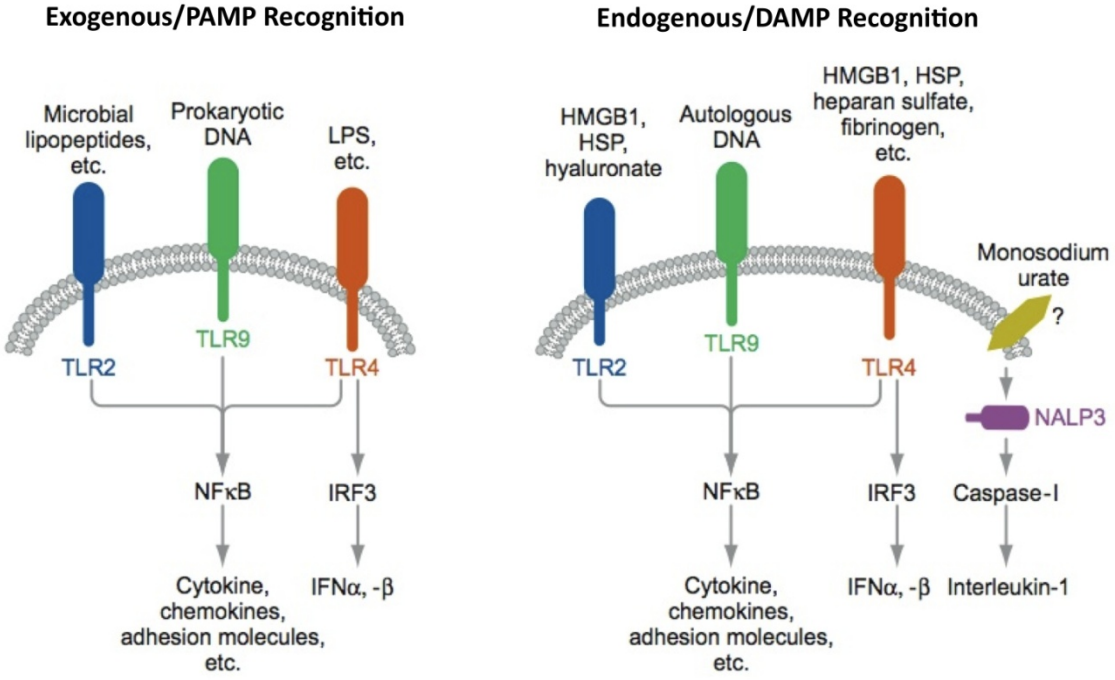

Figure 3-1: Exogenous (PAMP) and endogenous (DAMP) TLR recognition. TLRs respond to both exogenous (PAMPs) and endogenous (DAMPs) molecules through similar pathways following ligation, including NF-κB. Image adapted from (Rock and Kono 2008).

Due to the wide variety of putative 'danger signal' ligands, TLR4's role in the inflammatory response following sterile injury has also been investigated (Bettinger et al. 1994; Paterson et al. 2003; Barsness et al. 2004; Jiang et al. 2005; Mollen et al. 2006;

Kaczorowski et al. 2008; Sabroe et al. 2008; Zhang et al. 2008). For example, systemic inflammation as marked by pro-inflammatory cytokine (tumor necrosis factor (TNF)-α and IL-6) serum presence was found to be dependent on TLR4 following femur fracture (or sham surgery) (Levy et al. 2006). Also, endotoxin resistant mice (C3H/HeJ) have decreased TNF-α content surrounding a linear wound at one day which correlated to increased collagen production and wound strength at 7 days following wound in comparison to wild-type (C3H/HeN) mice (Bettinger et al. 1994). Neutrophil accumulation following bleomycin-induced acute lung injury depended on a combination of TLR4 and TLR2 via signaling through MyD88 (see section 3.5.1) (Jiang et al. 2005). Burn injury in mice induced increased sensitivity to TLR ligands suggesting that injury may also prime the responsiveness of TLRs (Paterson et al. 2003). Lastly, neutrophil and monocyte recruitment in the peritoneal cavity following injection of necrotic cells depended on MyD88, TLR4/TLR2 and/or IL-1R (Chen et al. 2007). Therefore, endogenous molecules released or accumulated following injury seem to induce and augment a pro-inflammatory host reaction and aid in directing the wound healing response.

Support for a 'danger signal'-induced adjuvant effect was found with the only clinically approved adjuvant 'alum', composed of insoluble aluminum salts, as well as PLGA microparticles which both induce IL-1β mediated DC inflammation via the intracellular nucleotide-binding oligomerization domain-like (Nod-like) receptor, NALP3, activation of caspase-1 (Li et al. 2007; Sharp et al. 2009). Furthermore, alum and PLGA particulate adjuvant effects through NALP3 are found to be dependent on the presence of 'danger signals' such as HSPs (Sharp et al. 2009).

Several integrins or CRs (see section 3.5.2) recognize endogenous molecules such as opsonizing complement components (e.g. C3b) as well as fibrinogen as part of the innate immune response to a pathogen or tissue damage, respectively (Wright et al.

1988; Gasque 2004). APCs, such as DCs, may use these integrins as both an instigator of antigen uptake and maturation to initiate an adaptive immune response (Carroll 2004). However, there is conflicting evidence since CR3 ($\alpha_M\beta$) ligation on DCs resulted in increased MHC class II and co-stimulatory molecule expression but decreased cytokine secretion (Behrens et al. 2007).

3.3. Host Response to a Biomaterial Implant

The host response to a biomaterial implantation includes sequentially, injury, blood/material interactions, provisional matrix deposition, acute and chronic inflammatory response, granulation tissue onset, foreign body reaction and fibrous encapsulation (Anderson et al. 2008) with durations of each stage dependent on biomaterial properties. The inflammatory response toward a material is inherently linked with injury to vascularized connective tissue (Anderson 2001). Initial blood/material interactions yield a deposited plasma protein profile on the material to subsequently direct the host response. Therefore, it is generally regarded that the host responds not to a biomaterial itself but to the adsorbed protein layer. Specifically, proteins which bind with higher affinity to a biomaterial will likely contribute to cellular recognition as over time these proteins remain adsorbed as dictated through the Vroman effect (Vroman and Adams 1969). Numerous plasma proteins have been found to a play a role, in part, in the biomaterial-mediated host response. These include, for example, fibrinogen (Tang and Eaton 1993; Hu et al. 2001; Tang et al. 2002; Zdolsek et al. 2007), plasminogen (Busuttil et al. 2004), fibronectin (Keselowsky et al. 2007), vitronectin (Wilson et al. 2005), various complement proteins (Gorbet and Sefton 2004), thrombospondin 2 (Kyriakides et al. 1999) and immunoglobulins (Anderson et al. 2008). The adsorbed protein profile deposited on the material surface directs recruitment and activation of

cells and subsequently leads to foreign body reaction and fibrous encapsulation depending on the material size.

Neutrophil and monocyte recruitment to the peritoneal cavity has been found to be induced by 'danger signal' presence (Chen et al. 2007) during an acute inflammatory response which persists at the site of injury/implantation temporarily for approximately 24hr (Anderson 2001). Following this response, macrophages, which differentiate from monocytes after leaving the vasculature, are recruited to the implant site and may maintain their presence for months (Anderson 2001). Macrophages are the dominant cell type believed to be responsible for directing the wound healing response to an implantation. They do so by secreting pro-inflammatory cytokines/chemokines (such as TNF-α) if classically activated (M1) (Duffield 2003) by TLR ligands. A balance between macrophage phenotypes at the site of injury has also been found in which M1 macrophages aid in clearance of necrotic cells and damaged matrix while macrophages alternatively activated (M2) by anti-inflammatory cytokines such as IL-4 help direct the wound healing response through further release of anti-inflammatory cytokines that in turn help induce vascularization, matrix deposition and induce proliferation of fibroblasts locally (Duffield 2003; Mosser and Edwards 2008). Fibroblasts produce primarily type I collagen (Anderson 2001) that forms both the initial granulation tissue (scar formation) and subsequent fibrous capsule which surrounds an implant (Anderson et al. 2008)

To study proteins and receptors involved in the host response to a biomaterial using genetically altered mouse models, implantation of polyethylene terephthalate (PET) (Dacron™) discs is often used as a model biomaterial implant (Tang et al. 1998; Hu et al. 2001; Tang et al. 2002; Busuttil et al. 2004; Keselowsky et al. 2007; Zdolsek et al. 2007). PET yields a robust acute inflammatory response at 16hr when implanted into the peritoneal cavity in mice eliciting high levels of phagocyte accumulation (neutrophils and monocytes) on the surface (Tang and Eaton 1993). When examining the chronic

host response, PET implanted subcutaneously for two weeks induces a typical fibrotic reaction (encapsulation) (Tang et al. 2002; Keselowsky et al. 2007).

3.4 Complement Pathway

Serum complement proteins opsonize foreign microbes/materials for clearance (Abbas et al. 2007). There are three main pathways for complement activation: the classical, the alternative and the lectin pathway (Abbas et al. 2007). The classical pathway involves complement protein C1 binding at least two opsonizing IgG molecules, while the alternative pathway involves C3 binding covalently (C3b) to the microbe or biomaterial surface directly through hydroxyl or amine groups (Gorbet and Sefton 2004). Lastly, the lectin pathway is activated by circulating lectins binding to carbohydrate moieties (particularly mannose) on the surface of microbes which in turn activates complement proteins C4b/C2 to covalently tag the microbe (Abbas et al. 2007). Irrespective of initiation, the complement activation pathways merge at the level of formation of a membrane attack complex (MAC) which serves to induce cell lysis of the microbe (Muller-Eberhard 1986). Biomaterial presence *in vivo* may trigger complement activation via the alternative pathway or the classical pathway as seen on chromium or titanium surfaces (Walivaara et al. 1996; Walivaara et al. 1996). DCs, and other leukocytes, possess specialized integrins (or CRs), which are capable of recognizing these complement-opsonized entities. Nanoparticle biomaterial vaccines have been successfully designed to take advantage of the complement pathway (through C3b binding to hydroxylated surfaces) to induce an increased immune response toward an associated antigen (Reddy et al. 2007).

3.5. Dendritic Cell Receptors: TLRs, Integrins, and Other Receptor Signaling

3.5.1 Toll-like Receptors

Toll-like receptors (TLRs), the most characterized of the PRRs, are integral membrane proteins containing varying amounts of leucine-rich repeats in their extracellular domains thought to be involved in the ligand-recognition process (Kawai and Akira 2006). Currently, twelve TLR family members have been discovered in mammals (Akira et al. 2006) which recognize a variety of bacterial and viral PAMPs. Examples of microbial or viral ligands for TLRs include LPS (TLR4) (Poltorak et al. 1998; Hirschfeld et al. 2000; Guha and Mackman 2001; Brunn et al. 2005) lipopeptides and lipoproteins (TLR2) (Hirschfeld et al. 2000), double-stranded viral RNA (TLR3) (Kawai and Akira 2006) and bacterial flagellin (TLR5) (Hayashi et al. 2001). TLRs such as TLR4, TLR2 and TLR5, which recognize extracellular pathogen components, are found across the plasma membrane while others such as TLR3 and TLR9, which recognize pathogen intracellular molecules, are localized to endosomes owing to necessary pathogen degradation prior to engaging the receptors.

As stated previously, TLRs (particularly TLR4) have also been shown to be involved in various 'danger signal'-mediated signaling. Most TLR signaling propagation occurs through MyD88, an adaptor protein common for nearly all TLRs (except TLR3). TLR4, however, is the only TLR that can signal in a MyD88 dependent and independent manner (Kaisho and Akira 2001). TLRs share cytoplasmic homology to IL-1 receptor (IL-1R), and thus the adaptor molecules and signaling pathways are also shared. In short, upon ligation TLRs stimulate three main signaling pathways: 1. IKK complex (inhibitor of nuclear factor-κB (IκB)-kinase complex) activation results in the phosphorylation of IκB (which subsequently becomes degraded via ubiquitinylation) and results in the release and activation of NF-κB transcription factor family (p65, p50, p52, RelB, and cRel)

normally sequestered in the cytoplasm; 2. Mitogen-activated protein kinase (MAPK) family (c-Jun N-terminal kinase, JNK; p38, and extracellular-signal-regulated kinase, ERK) activation results in the subsequent phosphorylation/activation of AP-1 transcription factor family (cJun, cFos, FosB, Fra-1, Fra-2, JunB, JunD); 3. MyD88-independent pathways signal through an adaptor molecule Toll/IL-1R domain-containing adapter molecule (Trif) which leads to the activation of interferon regulatory factor 3 (IRF3) transcription factor to induce interferon production (Kawai and Akira 2006) (Fig. 3-2). Though NF-κB and AP-1 independently control the expression of co-stimulatory molecules and inflammatory cytokines particularly in DCs, balanced activation of both NF-κB and AP-1 seems to yield optimal inflammatory response (Kriehuber et al. 2005; Papa et al. 2006). Upon activation of TLR4 by LPS, TLR4 surface expression is down-regulated making DCs hypo-responsive to subsequent LPS stimulation (Visintin et al. 2001).

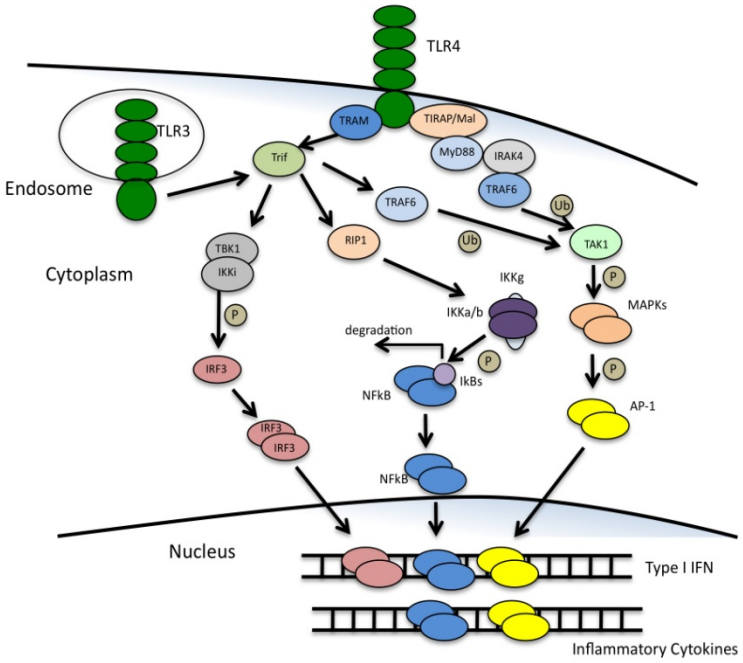

Figure 3-2: Representative TLR signaling pathways. TLR4 and TLR3, shown. Image adapted from (Kawai and Akira 2006). TLR mediated responses yield typical inflammatory responses through the activation of NF-κB and AP-1 transcription factors.

In regards to biomaterial-mediated responses, TLR4, in particular, has been shown to play a role in the inflammatory reaction to hydroxyapatite particles (Grandjean-Laquerriere et al. 2007) and alginate oligosaccharides (Iwamoto et al. 2005) in macrophages. Also, TLRs expressed by several cell types (e.g. endothelial cells, macrophages) are found in the interface membrane of loosening-hip implants (Takagi et al. 2007). Intracellular Nod-like receptors, which possess similar leucine-rich repeats to TLRs, have been found to account for PLGA MP induced adjuvanticity through DC inflammasome activation (Sharp et al. 2009). Recently, DCs treated with various biomaterials were found to induce signs of DC maturation and subsequent T cell stimulation in a TLR-dependent manner as DCs from MyD88$^{-/-}$ or TLR4$^{-/-}$ mice show

deficiencies in their response to biomaterials (Shokouhi et al. 2010). Thus, TLRs are relevant receptors for investigation into the DC recognition of biomaterials.

3.5.2 Integrins

Integrins are trans-membrane adhesion receptors composed of heterodimers of at least 24 combinations of distinct α and β subunits, which mediate cell-cell adhesion and adhesion to surrounding tissues through binding of extracellular matrix proteins to support cell migration (Abram and Lowell 2009). Families of integrins are often designated by a common β subunit and differ in their ligand specificity through the assortment of α subunits. Integrins are capable of recognizing a wide variety of physiological ligands such as fibronectin, vitronectin and fibrinogen as well as many cell surface molecules (Arnaout et al. 2005). While many cell types possess β_1 integrin family members, only leukocytes, including DCs, possess the β_2 (or CD18) family of integrins (Springer 1990; Ginsberg et al. 1992; Dib and Andersson 2000). β_2 is known to pair with four α subunits (α_L, α_M, α_X or α_D) and is also known to mediate responses to infection or inflammation by supporting transmigration through endothelium by leukocytes (Gahmberg et al. 1998). Inflammation-induced activation of leukocytes via chemokines is capable of inducing an inside-out signaling transformation of integrins (Rose et al. 2007) to a high affinity confirmation capable of supporting binding to its ligand. Activated leukocytes have increased binding capacity of integrins due to shifting the integrin state from a closed to an open form (Abram and Lowell 2009). Once integrins bind to a ligand, integrin clustering occurs and further leads to adhesion-induced cell signaling (Rose et al. 2007).

Expressed on human DCs, β_2 family members include $\alpha_L\beta_2$ (lymphocyte function-associated antigen, LFA-1), $\alpha_M\beta_2$ (or CR type 3, CR3; macrophage-1 antigen, MAC-1) or

$\alpha_X\beta_2$ (CR type 4, CR4) (Bajtay et al. 2006). β_2 integrins such $\alpha_M\beta_2$ or $\alpha_X\beta_2$ may facilitate host recognition of both complement-opsonized microbes through inactivated (i)-C3b or biomaterials through adsorbed fibrinogen (Lee and Kim 2007). Monocyte adhesion to biomaterials is dependent on C3-opsonization and $\alpha_M\beta_2$ (McNally and Anderson 1994; McNally and Anderson 2002). β_2 integrins have also been found to recognize biomaterial-adsorbed fibrinogen and explain, in part, the host recognition of biomaterials (Tang and Eaton 1993; Tang et al. 1996; Tang et al. 1998). Regardless, β_2-mediated adhesion to biomaterials by mononuclear cells such as monocytes/macrophages is believed to be important in the initiation of the host response (Anderson 2001).

Integrin-ligand binding leads to outside-in signaling which may further maintain the activation state of the integrin and lead to both actin polymerization and gene transcription. Kinases involved in the integrin signaling are Src, focal adhesion kinase (FAK) and spleen tyrosine kinase (Syk) which is recruited by phosphorylated immunoreceptor tyrosine-based activation motifs (ITAMs) (Fig. 3-3) (Abram and Lowell 2009). Furthermore, a link between an integrin signaling pathway and TLRs has been shown. α_M induced signaling results in Syk phosphorylation, which in turn directly phosphorylates MyD88 and Trif and marks them for ubiquitin addition (Han et al. 2010). This process leads to proteasomal degradation of the two key adaptor molecules for TLR signaling. Therefore, integrin α_M engagement leads to an inhibition in TLR-induced activation (Han et al. 2010).

The role of integrins for DC biomaterial recognition is less characterized, but similar to monocyte/macrophages DC adhesion to distinct plasma proteins, which is believed to be integrin mediated, does contribute to signs of maturation (Acharya et al. 2008). Also, it is known that biomaterial-adsorbed fibrinogen induces DC cytokine secretion in a β_2-dependent manner (Thacker and Retzinger 2008). Furthermore, as

stated previously $\alpha_M\beta_2$ ligation in DCs induces increased co-stimulatory and MHC class II molecule expression while decreasing cytokine secretion (Behrens et al. 2007).

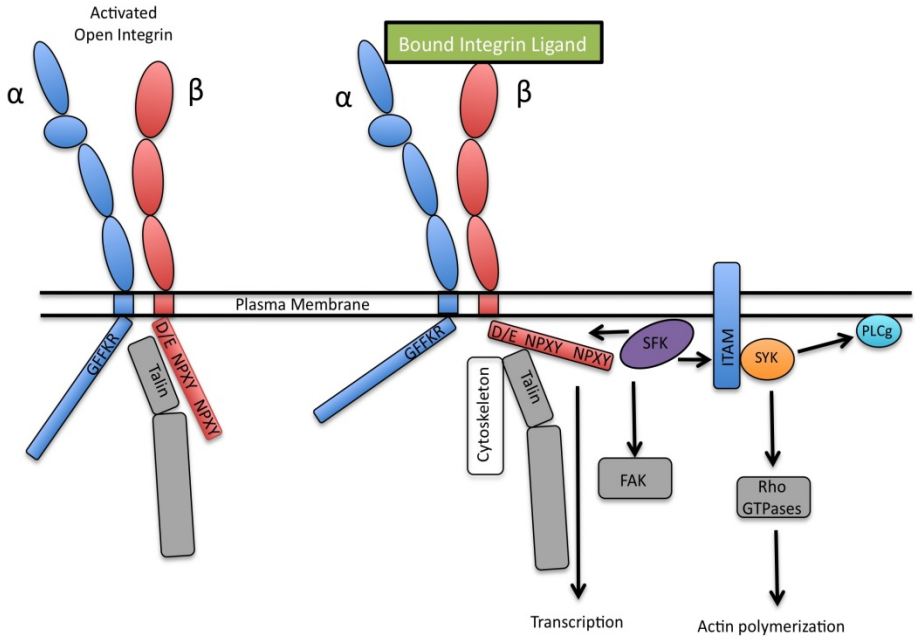

Figure 3-3: Active integrin signaling pathway, adapted and simplified from (Abram and Lowell 2009). Integrin-ligand recognition results in activation Src family kinases (SFK), ITAMs, talin (which is directly bound to cytoskeleton), and also FAK. Integrin binding leads to both transcription and actin polymerization.

3.5.3 C-type Lectins

C-type lectins receptors (CLRs) contain carbohydrate recognition domains (CRDs) which depend on Ca^{++} for both ligand binding and maintaining structural integrity (Cambi et al. 2005). CLRs recognize carbohydrate moieties present on pathogens but also altered carbohydrate structures found on apoptotic and necrotic cells (Gijzen et al. 2006) that are marked for clearance. On pathogens CLRs recognize specific carbohydrates such as mannose structures using the mannose receptor (MR) family which facilitate uptake and antigen processing. Other family members of C-type lectins

include collectins, and type II receptors such as dendritic cell-specific ICAM3 grabbing non-integrin (DC-SIGN). DC-SIGN is involved in binding to intercellular adhesion molecule-3 (ICAM-3) on T-cells which helps mediate DC induced T-Cell activation (Figdor et al. 2002). Similar to TLRs, C-type lectins have been implicated in 'danger signal' interactions by recognizing apoptotic cells with modified carbohydrate residues (Peiser et al. 2002; Gijzen et al. 2006). The signaling pathways of lectins are less characterized than TLRs though some pathways have been shown. Dectin-1, a type II receptor, binding by ß-glycan has been found to stimulate NF-κB activation through its intracellular ITAM and subsequent spleen tyrosine kinase (Syk) activation (Brown 2006; Gijzen et al. 2006). The role of C-type lectins in recognizing endogenous/aberrant glycosylations found in association with biomaterial surfaces is unknown but is also being investigated in the Babensee laboratory.

3.5.4 Scavenger Receptors

Scavenger receptors (SR) consist of a wide range of integral membrane proteins involved in the recognition of polyanionic ligands (Peiser et al. 2002) and were originally believed to mediate endocytosis of oxidized low-density lipoproteins (Abbas et al. 2007). Similar to CLRs and TLRs, SRs have recently been implicated in pathogen recognition and the clearance of apoptotic cells (Peiser et al. 2002). SRs (such as CD91 and SR-A) respond to endogenous intracellular HSPs such as HSPgp96, HSP70 and HSP90 (Facciponte et al. 2005) many of which have been found to stimulate TLR signaling.

3.5.5 Fc-Receptors

Dendritic cell Fc-receptors recognize fragment crystallizable (Fc) regions of host immunoglobulin molecules that opsonize antigens and help mediate phagocytosis. Depending on the presence of ITAM or immunoreceptor tyrosine-based inhibition motifs

(ITIM), Fc-receptors are grouped into two classes: activating or inhibiting receptors, respectively. ITAM-containing receptors can induce DC maturation through NF-κB activation while ITIM-containing receptors block ITAM-mediated maturation (Bajtay et al. 2006). ITAM-mediated signaling by Fc-receptors, similar to CLRs, is through Syk (Nimmerjahn and Ravetch 2008). Since immunoglobulin opsonization occurs on the surface of biomaterials (Anderson et al. 2008), it is foreseeable that Fc-receptors on DCs may mediate the recognition of a biomaterial and may even coordinate with CLRs through ITAM-containing based signaling.

3.6. Biomaterials Used in Combination Products

3.6.1 PLGA

Poly (lactic-*co*-glycolic) acid (PLGA) is a synthetic co-polymer with biocompatible/natural by-products widely utilized in the field tissue engineering as for cell scaffolds. Lactic and glycolic acid are covalently linked via an ester bond, which allows for direct hydrolysis *in vivo* and *in vitro* yielding its biodegradability (Kim and Mooney 1998). The degradation rate of PLGA may be fine-tuned by increasing glycolide content of the polymer (i.e. increasing the ratio of glycolide to lactide) (Wu and Ding 2004). Often mole ratios between 50:50 to 85:15 (lactide:glycolide) are used due to preferable *in vivo* half-lives of scaffolds which range from 2 weeks for 50:50 PLGA to 12 weeks for 85:15 PLGA (Lu et al. 2000). An appropriate amount of time is needed in order to allow for tissue integration, which will depend on the particular application and location in the body. For PLGA, perspective tissues range from bone and cartilage to liver tissues (Kim and Mooney 1998). Due to its natural base components, PLGA by-products are biocompatible and thus used in a wide variety of FDA approved devices.

PLGA may also be used as a controlled release delivery vehicle in the form of MP (or particulate), which gradually release their loaded components, such as proteins during bulk hydrolysis (Blanco and Alonso 1998). PLGA MPs have also been utilized as both vesicle and adjuvant as they may increase an immune response to the delivered product (Ertl et al. 1996; Bennewitz and Babensee 2005; Schlosser et al. 2008; Sharp et al. 2009) potentially through intracellular DC inflammasome activation (Sharp et al. 2009). However, the biomaterial adjuvant effect toward a co-delivered antigen has also been seen with PLGA in scaffold form which cannot be internally processed (Matzelle and Babensee 2004; Bennewitz and Babensee 2005); although microparticles are degradation products of the scaffolds upon contact with the aqueous environment *in vivo*. Therefore, further elucidating the host response to PLGA is necessary to understand its adjuvant effect.

3.6.2 Agarose

Agarose is a natural seaweed-derived polysaccharide containing disaccharides of D-galactopyranose and 3,6-anhydro-L-galactopyranose capable of forming thermally reversible physically-crosslinked gel (Lee and Mooney 2001; Liang et al. 2006). Agarose scaffolds and/or hydrogels have been used for tissue engineered devices ranging from nerve repair (Balgude et al. 2001; Stokols et al. 2006) to cartilage replacement strategies (Rahfoth et al. 1998). Due to its hydrophilic nature, it has an extremely low contact angle (Yoshida and Babensee 2006). Unlike PLGA scaffolds, agarose scaffolds do not contribute to an adjuvant effect toward a co-delivered antigen and maintain a humoral immune response equivalent to that of injection of antigen alone (Matzelle and Babensee 2004; Norton et al. 2010). Unlike other natural polymers used in tissue-engineered devices such as hyaluronan (Leach et al. 2004) which consist inherently in

their chemistry of receptor-recognizable motifs e.g. via CD44 or integrins, agarose does not possess a carbohydrate moiety recognizable by cellular receptors.

3.7. Biomaterial-induced DC Maturation

The relationship between innate and adaptive immunity in the context of a combination product, which elicits a response from both branches of the immune system (non-specific innate and antigen-specific adaptive response), has only recently been investigated. It was believed that the innate response toward a biomaterial might influence the subsequent adaptive immune response toward the co-delivered biologic. PLGA scaffolds, commonly utilized in tissue-engineered devices (Section 3.6.1), have been found to act as an adjuvant toward a co-delivered model antigen (ovalbumin, OVA) (Matzelle and Babensee 2004; Bennewitz and Babensee 2005; Norton et al. 2010). The implantation of the material scaffold (as opposed to the injection of PLGA MP) in association with antigen induced a higher and longer lasting humoral immune response (antibody production) to the model antigen possibly due to tissue injury induced endogenous adjuvants presence to prime the implantation site for a biomaterial adjuvant effect (Matzelle and Babensee 2004; Bennewitz and Babensee 2005; Norton et al. 2010). Upon tissue injury, DAMP presence increases (Section 3.2), which may aid in the propagation of an innate immune response through TLR recognition of molecules such as HMGB1 being released from cells or production of fragments of extracellular molecules such as FN. In fact, HGMB1 is found in higher presence in tissue exudates from subcutaneously implanted PLGA scaffolds (with or without OVA) in comparison to naïve controls suggesting its potential role in biomaterial-induced adjuvant effect (Babensee 2008).

It was hypothesized that DCs play a part in the biomaterial adjuvant effect as DCs are innate immune cells which serve to initiate an adaptive immune response. To

this extent, human monocyte-derived DCs were treated with biomaterials commonly used in tissue-engineering applications. It was found that DC maturation occurred in the presence of films prepared of materials such as PLGA and chitosan, did not occur in the presence of agarose films, and DC maturation was inhibited by treatment with hyaluronic acid films (Babensee and Paranjpe 2005). Also, treatment of DCs with PLGA or agarose films induced NF-κB activation in human DCs (Yoshida and Babensee 2006) though agarose seems to induce higher levels than PLGA film treatment. BMDCs from C57BL/6 mice have also been shown to mature in the presence of PLGA films or MP (Yoshida et al. 2007). PLGA film induction of DC maturation *in vitro* matches its adjuvanticity *in vivo*. Similarly, the minimal DC response to agarose film *in vitro* mimics the lack of agarose scaffold adjuvant effect (Matzelle and Babensee 2004; Bennewitz and Babensee 2005; Norton et al. 2010). Therefore, the response of DCs to the biomaterial component of a combination product may be critical and utilized to manipulate the subsequent host immune response.

CHAPTER 4

THE ROLE OF 'DANGER SIGNAL'-TLR4 INTERACTIONS IN THE HOST RESPONSE TO BIOMATERIAL IMPLANT [*]

Introduction

Biomaterials used for medical devices elicit a response from the body that is often detrimental to the efficacy of their intended function. Simply, the host response to a biomaterial implantation includes injury, blood/material interactions, coagulation and provisional matrix deposition, acute inflammatory response, granulation tissue onset, foreign body reaction and fibrous encapsulation (Anderson et al. 2008). The implantation procedure inherently produces tissue and vasculature damage causing blood components to infiltrate the implant region. The inflammatory response toward a device is directed by these interactions between blood/tissue components and material, which results in immediate protein adsorption to the surface.

A variety of proteins mediate the localization of leukocytes to biomaterials through adhesion receptors (Wilson et al. 2005). Particularly, complement proteins (McNally and Anderson 1994; Brodbeck et al. 2003; Gorbet and Sefton 2004) as well as a major component of the clotting cascade, fibrinogen (Tang and Eaton 1993; Tang et al. 1998; Hu et al. 2001), have been shown to mediate phagocyte adhesion to materials through β_2 (CD18) integrins (McNally and Anderson 1994; Tang et al. 1996; Hu et al. 2001; McNally and Anderson 2002). This adhesion is a critical step in the recognition of a biomaterial. However, there are potentially additional initial events in the inflammatory response to an implant that may prime phagocytes such as neutrophils and monocyte/macrophages for adhesion. Recent literature has brought to light the role of 'danger signals' in the inflammatory response following injury, which elicit leukocyte

[*] Modified from Rogers T, Babensee JE "Altered adherent leukocyte profile on biomaterials in Toll-like receptor 4 deficient mice" *Biomaterials*, 2010 31(4) 594-601

activation primarily through binding to pattern recognition receptors (PRRs). These ligand-receptor interactions would precede (or occur simultaneously with) leukocyte adhesion, but the role of these factors in the host response to a biomaterial is unknown.

'Danger signals' or damage-associated molecular patterns (DAMPs) (Matzinger 1994; Matzinger 2002) are endogenous intracellular or extracellular components that are normally hidden from the environment or are present at low concentrations. Upon cellular necrosis and/or tissue damage, these occult motifs can become strong activators of inflammation, aid in the body's recognition of dying cells and can act as adjuvants through direct activation of immune cells such as dendritic cells (DCs) and macrophages (Gallucci et al. 1999). Numerous endogenous DAMPs have been linked to leukocyte activation through the PRR Toll-like receptor 4 (TLR4) (Rock and Kono 2008) as well as the PAMP lipopolysaccharide (LPS).

The TLR family is composed of at least ten TLRs (TLR1-TLR10) in humans (Akira et al. 2006) and are integral membrane proteins containing leucine-rich repeats in the extracellular domain which are believed to be involved in the ligand-recognition process. Immune cells (along with other cell types) possess TLRs, which canonically bind conserved pathogen-associated molecular patterns (PAMPs) to aid in the initiation of an innate immune response toward a pathogen (Medzhitov and Janeway 1997; Janeway and Medzhitov 2002; Lee and Kim 2007). TLR activation stimulates innate effector functions such as increases in pro-inflammatory cytokine/chemokine production (e.g. TNF-α), increases in co-stimulatory molecules for antigen-presenting cells, and up-regulation of integrin expression (Sabroe et al. 2003). TLR4/DAMP interactions have been investigated in the wound healing response to sterile injury in various applications ranging for hemorrhage and drug-induced lung injury to sterile skin incisions and systemic inflammation following femur fracture (Bettinger et al. 1994; Barsness et al. 2004; Jiang et al. 2005; Levy et al. 2006). Following injury, it is believed that TLR4

initiates inflammatory signaling via cytokine communication (Mollen et al. 2006) to provide necessary cues during wound repair. Neutrophil and monocyte recruitment to the peritoneal cavity has been found to be induced by DAMP presence (Chen et al. 2006). These findings are relevant to biomaterials as many aspects of wound healing are connected to the host response to materials (Anderson 2001; Anderson et al. 2008).

Biomaterial particulates delivered with PAMP ligands for TLR4 have been investigated for directing cancer vaccines (Hamdy et al. 2008; Schlosser et al. 2008); however, we and others have found that biomaterials themselves are capable of acting as adjuvants inducing a strong humoral immune response toward a co-delivered antigen (Matzelle and Babensee 2004; Bennewitz and Babensee 2005; Babensee 2008; Sharp et al. 2009) in the absence of PAMP TLR ligands. Therefore, in an endotoxin-free environment, TLR4 may interact with biomaterial-associated DAMPs to propagate an innate inflammatory response and offers an explanation of the biomaterial-induced adjuvant effect previously seen.

Several groups have studied the role of TLR4 in the cellular response to biomaterials *in vitro*. Macrophage cytokine secretion in response to oligosaccharides of alginate is believed to be TLR4/2 dependent (Iwamoto et al. 2005). Pro-inflammatory cytokine production (TNF-α) but not NF-κB activation in macrophages treated with hydroxyapatite particles was found to be TLR4-dependent (Grandjean-Laquerriere et al. 2007). However, as to date no studies have examined the role of TLRs (specifically TLR4) in the *in vivo* host response to biomaterials. Therefore, this study sought to examine the role of TLR4 and endogenous DAMP molecule interactions in the acute and chronic inflammatory response to a biomaterial implant.

Materials and Methods

Biomaterial Preparation

PET implants were prepared by punching 10mm discs from a 0.5mm thick PET sheet (AIN Plastics, Kennesaw, GA). Discs were sterilized by rinsing with 70% ethanol for 24hr followed by three 30 min rinses in endotoxin-free water (Lonza, Basel, Switzerland). After drying in a tissue culture hood, discs were autoclaved prior to implantation. Endotoxin content of equivalently prepared PET discs (5mm) was assessed using the Limulus Ameobocyte Lysate (LAL) assay (QCL-1000 Chromogenic LAL Endpoint assay, Lonza) in endotoxin-free test tubes (Lonza). PET discs were tested in the presence of both LAL and the chromogenic substrate and compared to a simultaneously developed endotoxin standard curve (between 0.1 and 1.0 EU/mL). Implanted PET discs tested below 0.1 EU/ml, the lower limit for this assay.

IP Implantation and Analysis

All animal procedures were approved by IACUC committee prior to use (Protocol 044-2008, Emory University). Mice were 6-7 weeks old and age-matched for each experiment performed across $TLR4^+$ (C57BL/10, The Jackson Laboratory, Bar Harbor, ME) and $TLR4^-$ (C57BL/10ScN, The Jackson Laboratory) strains. For IP implantation model, survival surgeries were completed in a laminar flow hood using sterilized surgical instruments in combination with a peri-operative dose of nalbuphine (6mg/kg) under isofluorane (Baxter, Deerfield, IL) anesthetic. Two PET discs were implanted IP per mouse for 16hr through a midline incision followed by suturing of muscle and skin (n = 7-9 mice, see Table 4-1). Sham surgeries with no implants (but with incision and suturing) were also performed simultaneously (see Table 4-1). At 16hr, mice were anesthetized and 1ml Dulbecco's Phosphate Buffered Saline (Invitrogen, Carlsbad, CA) [D-PBS]/heparin (Abraxis Bioscience, Los Angeles, CA) (50 U/ml) was injected IP for

collection of lavage by sterile transfer pipette. Discs were collected and transferred to 1ml PBS on ice until analysis. Lastly, cardiac puncture (using 100 U heparin preloaded in a 1mL syringe with a 21g needle) was performed to subsequently determine circulating leukocyte population.

Lavage supernatants were collected, volume estimated for concentration calculation and stored at -20^{o}C following centrifugation of lavages at 400g for 5 min. Lavage cells were resuspended in 1ml of D-PBS and a small aliquot (10µl) was analyzed for cell counts (6-20µm) using Multisizer 3 Coulter Counter (Beckman Coulter, Fullerton, CA). The remaining cells were resuspended at a concentration of 1*10^{6} per milliliter and

Table 4-1: Number of animals (n) analyzed per strain using the indicated endpoint analyses for the three treatment groups in the IP implantation study. Lavage total cell counts and cytospin analysis were performed on all mice including those with PET implants which were also analyzed for total adherent cells and cell types (see method for detailed description). Cardiac punctures were performed on all mice to assess circulating leukocyte populations

Treatment	*Strain*	*Analysis*	*n*
Naïve	TLR4^{+}	Lavage	7
	TLR4^{-}	Lavage	8
Sham	TLR4^{+}	Lavage	9
	TLR4^{-}	Lavage	7
Implant	TLR4^{+}	Lavage	9
	TLR4^{-}	Lavage	9
	TLR4^{+}	Implant	8
	TLR4^{-}	Implant	9

200µL used for one cytospin analysis per mouse/lavage using Shandon Cytospin 3 (Thermo Scientific, Waltham, MA). Cytospins were stained with Hema 3 (Fisher

Scientific, Pittsburgh, PA) and differential cell counts (300 cells counted per sample) performed by microscopy.

The first explanted disc was stained immediately after recovery using Hema 3. From the second recovered disc, adherent cells were collected by placing the disc in trypsin/EDTA (0.25%) (Sigma-Aldrich, St. Louis, MO) at 37^{o}C. To assure complete removal of adherent cells, a cell scraper was lightly passed over both sides of the disc after it had been placed in RPMI 1640 media (Invitrogen) supplemented with 10% heat-inactivated FBS (Mediatech, Manassas, VA) to inactivate trypsin. Collected adherent cells were counted and stained using the identical procedure described previously for lavage cells (one cytospin analysis per disc, n = 8-9 samples per group, see Table 4-1). This implant was stained directly to assure complete removal of cells had been achieved. The trypsinized disc possessed no adherent cells on either side compared to the implant stained immediately after removal which was extensively covered with cells as assessed by microscopy.

Cardiac punctures were collected and immediately a blood smear prepared using HemaPrep (J.P. Gilbert Company, Boyertown, PA) and stained using Hema 3. Red blood cells were lysed with warmed ammonium chloride solution to assess total circulating leukocyte concentrations using a Multisizer 3 Coulter Counter (Beckman Coulter).

Naïve lavages and cardiac punctures were also performed in age-matched TLR4^{+} and TLR4^{-} mice that had not undergone any surgical procedure (n = 7-8 mice, see Table 4-1).

SC Implantation and Analysis

For SC implantation model, survival surgeries were completed in a laminar flow hood using sterilized surgical instruments in combination with peri-operative dose of nalbuphine (6mg/kg) and isofluorane (Baxter) anesthetic. Two PET discs were implanted subcutaneously on the dorsum (one to the left and one to the right of the spine) of $TLR4^+$ (n=6) or $TLR4^-$ (n=7) mice. At 2 weeks, mice were anesthetized using isofluorane and cardiac punctures performed/analyzed as described above. Discs with surrounding tissue were explanted and immediately fixed in 10% (v/v) buffered formalin (Fisher Scientific) until processing. Tissue explants were embedded in paraffin and 5μm sections were prepared for staining. For analysis of fibrous capsule thickness, sections were deparaffinized and stained with Van Gieson stain (American Master Tech Scientific, Lodi, CA); otherwise, sections were stained with hematoxylin and eosin (H&E). Fibrous capsule thickness (as seen by dark red collagen staining) was determined on both skin or muscle side of implants from five sections per mouse using Image Pro Plus (Media Cybernetics, Bethesda, MD) calibrated software using sections taken vertically from the center of the explanted disc.

TNF-α ELISA Analysis

Cleared lavage supernatants from implant, sham or naïve treatment groups were assessed for TNF-α concentration using an ELISA (R&D Systems, Minneapolis, MN). Samples were run undiluted and analyzed following manufacturer's instructions. Values that were below blank of the assay were taken as zero.

Statistical Analysis

All statistical analysis was performed using one-way ANOVA (Prism v5.0a, GraphPad Software Inc, La Jolla, CA) with animals nested within treatment. Comparison

across groups was accomplished using a Tukey post-test with a 95% confidence interval ($p<0.05$ was considered significant).

Results

Using an established TLR4-deficient mouse strain, C57BL/10ScN (Poltorak et al. 1998; Netea et al. 2003; Paterson et al. 2003; Takeda et al. 2003; Netea et al. 2005; Khan et al. 2006), which possesses a complete deletion of the *tlr4* gene, along with an appropriate wild-type control strain (C57BL/10) as recipients of biomaterial implants, the role of TLR4 in the host response to a biomaterial implant was examined. Discs of PET, as a model synthetic implant, have been used extensively (Tang et al. 1998; Hu et al. 2001; Tang et al. 2002; Busuttil et al. 2004; Keselowsky et al. 2007; Zdolsek et al. 2007) and yield robust phagocyte accumulation (neutrophils and monocyte/macrophages) in response to biomaterial in the peritoneal cavity at 16hr (Tang and Eaton 1993). To study the role of DAMPs/TLR4 interactions in the acute inflammatory response to biomaterials, PET discs were implanted IP for 16hr in $TLR4^-$ or $TLR4^+$ mice and total leukocyte concentrations and differential leukocyte profiles were analyzed in IP lavages and adherent cells harvested from the PET discs. Sham surgeries and naïve mice were used as controls to isolate the role DAMPs/TLR4 interaction plays in the recruitment of leukocytes due to the biomaterial itself. The dependence of the fibrous capsule formation on DAMPs/TLR4 interactions was assessed following 2 weeks of PET disc implantation subcutaneously in $TLR4^-$ or $TLR4^+$ mice.

Total leukocyte concentrations were determined in IP lavages collected from $TLR4^+$ or $TLR4^-$ mice receiving a PET disc implant, sham surgery or in the naïve control group (Fig. 4-1). Implantation of a PET disc induced recruitment of leukocytes into the peritoneal cavity of $TLR4^-$ mice with higher total leukocyte concentrations as compared to that of the respective naïve mice. This trend was also present upon PET disc implantation into $TLR4^+$ mice but was not found to be statistically significant. Sham surgery induced leukocyte recruitment into the peritoneal cavity of either mouse strain to

levels which were between those found for naïve or implant groups of either mouse strain, though not-statistically different from either group (Fig. 4-1).

The differential leukocyte profiles in IP lavages for $TLR4^+$ or $TLR4^-$ mice were determined and found to be similar for both mouse strains for the various treatment groups (Fig. 4-2). The majority of leukocytes in the peritoneal cavity at 16hr were neutrophils for both mouse strains regardless of treatment; however, there was a significantly lower fraction of neutrophils in naïve $TLR4^-$ than all $TLR4^+$ treatments (Fig. 4-2A). $TLR4^-$ mice tended to have a slightly higher fraction of monocyte/macrophage than $TLR4^+$ mice (Fig. 4-2B); however, no significant differences were found between

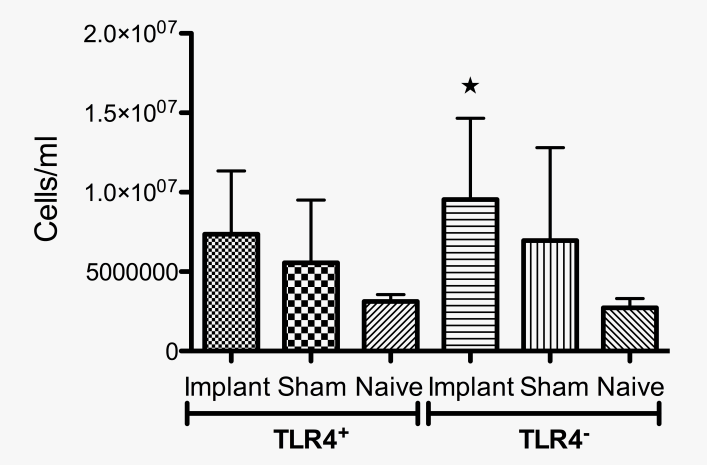

Figure 4-1: Total leukocyte concentrations in IP lavages for $TLR4^+$ or $TLR4^-$ mice. Mice received a PET disc implant, sham surgery or were in naïve control group. Star; $p<0.05$ in comparison to either naïve group, n=7-9 mice per group. Bars represent mean+SD.

implant groups of the two strains. Eosinophil and lymphocyte fractions from both strains were found to be similar (Fig. 4-2C, 4-2D, respectively). To examine if a TLR4 deficiency affected circulating leukocyte concentrations, which would certainly have affected recruitment into the IP space, total circulating leukocyte concentrations were determined

in lysed whole blood obtained from cardiac punctures. No differences were found across all treatments (data not shown) for both $TLR4^+$ and $TLR4^-$ mice.

As a potent pro-inflammatory cytokine often produced upon TLR4 signaling, TNF-α controls numerous cellular actions in the acute inflammatory response. The concentration of TNF-α in peritoneal cavity was determined to assess if it was induced in a TLR4-dependent manner following biomaterial implantation for 16hr. TNF-α was only

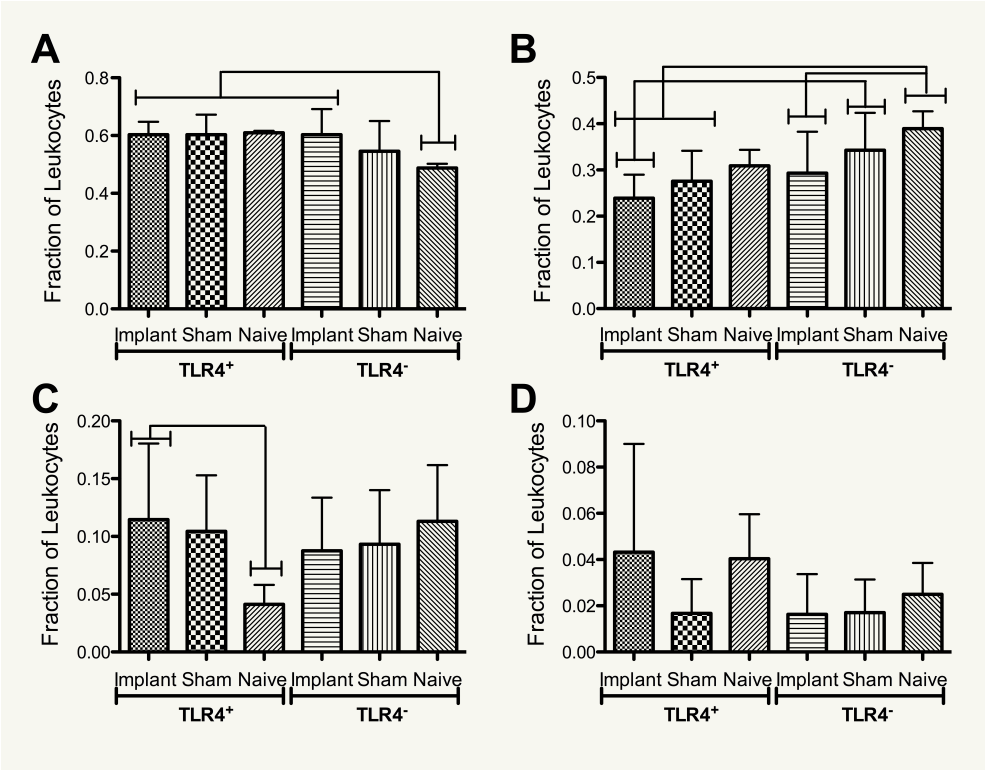

Figure 4-2: Differential leukocyte profiles for $TLR4^+$ or $TLR4^-$ mice. Fractions of (A) Neutrophils, (B) Monocyte/Macrophages, (C) Eosinophils, (D) Lymphocytes in IP lavages for $TLR4^+$ or $TLR4^-$ mice receiving a PET disc implant, sham surgery or naïve control. Brackets indicate significant differences between groups ($p<0.05$), n=7-9 mice per group. Bars represent mean+SD.

significantly detected in the peritoneal cavity upon implantation of a PET disc since little to no TNF-a was detected in IP lavages of sham or naïve groups from both $TLR4^+$ and $TLR4^-$ mice (Fig. 4-3). However, this response was found to be TLR4-independent as both strains elicited similar production of the cytokine upon PET disc implantation.

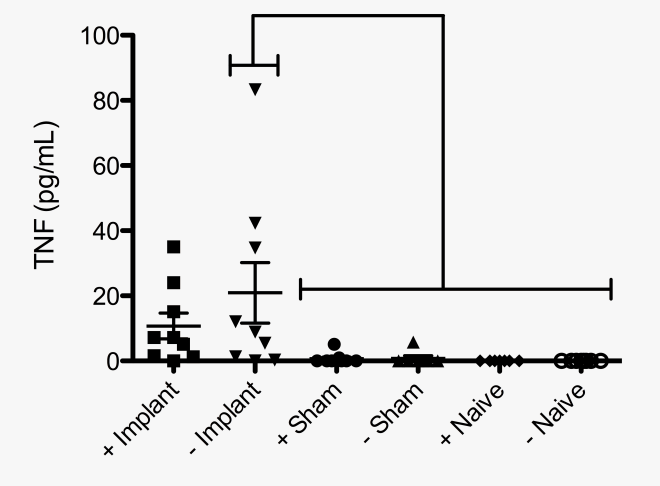

Figure 4-3: TNF-α concentrations in IP lavages for $TLR4^+$ (+) or $TLR4^-$ (-) mice receiving a PET disc implant (Implant), sham surgery (Sham) or naïve control (Naïve). Individual murine concentrations are plotted as points with horizontal lines representing mean with standard deviations. Brackets indicate statistical difference ($p<0.05$) between group, n=7-9 mice per group.

The role of TLR4 in controlling the adherent leukocyte profile on the IP implanted PET disc was assessed by determining the total number and differential profiles of leukocytes harvested by trypsinization from discs. The total numbers of leukocytes recovered as adherent to the biomaterial were similar in $TLR4^+$ and $TLR4^-$ mice (Fig. 4-4A). However, the differential leukocyte profiles recovered from the surface of the material were distinct between strains (Fig. 4-4B). Implants in $TLR4^+$ mice possessed equivalent fractions of harvested adherent neutrophils and monocyte/macrophages while implants in $TLR4^-$ mice had significantly higher fractions of harvested adherent

neutrophils than monocyte/macrophages (Fig. 4-4B and Fig. 4-5). Furthermore, both of these fractions were significantly different between the two strains (Fig. 4-4B).

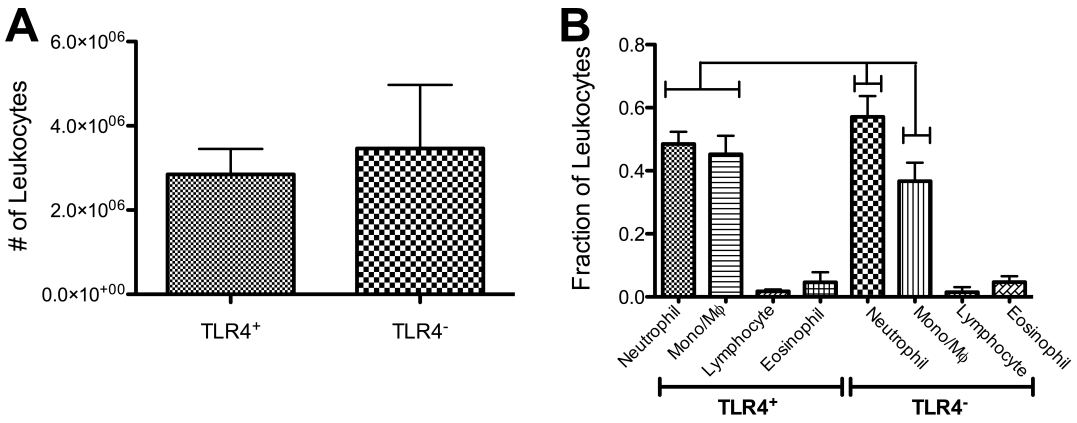

Figure 4-4: Adherent leukocyte profiles on PET discs following 16hr of IP implantation into TLR4+ or TLR4- mice. (A) Total number of adherent leukocytes collected from implants in either strain. No statistical difference was found between strains ($p=0.27$); n=7-9 mice per group. (B) Adherent leukocyte profiles showing fractions of neutrophils, monocyte/macrophages, eosinophils, and lymphocytes. Brackets indicate significant differences between groups ($p<0.05$); n=7-9 mice per group. All treatments significantly different from each other except for lymphocyte and eosinophil fractions from either strain as well as neutrophil and monocyte/macrophage fractions from TLR4$^+$ strain.

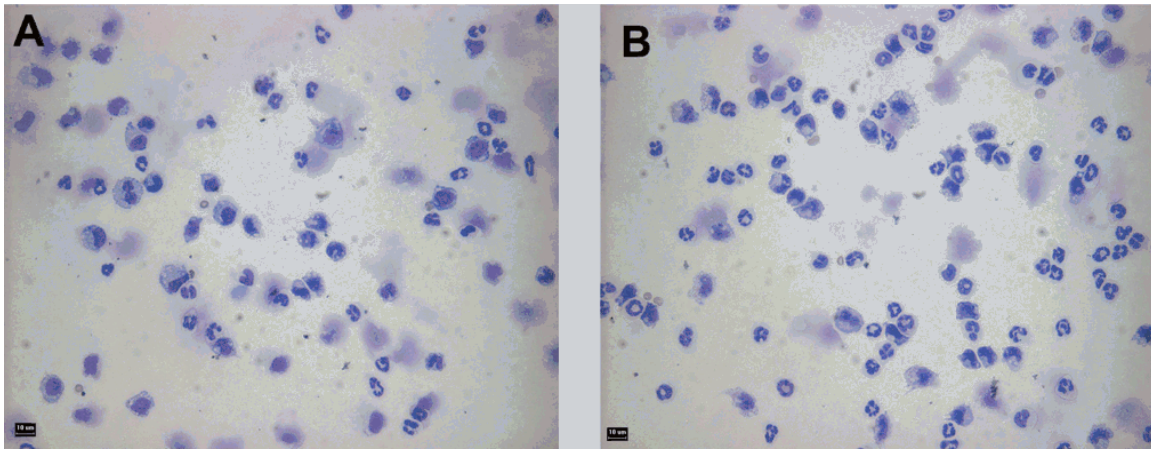

Figure 4-5: Representative cytospins of adherent leukocytes collected from PET discs implanted for 16hr. Found in TLR4$^+$ (A) or TLR4$^-$ (B) mice. Bar indicates 10μm.

To examine whether TLR4 presence affected the fibrous encapsulation of an implanted biomaterial, PET discs were implanted subcutaneously in $TLR4^+$ or $TLR4^-$ for 2 weeks and the resultant thicknesses assessed in histological sections. Tissue surrounding the implants was stained with Van Gieson to highlight the collagen content of the capsule. $TLR4^+$ and $TLR4^-$ mice displayed no noticeable differences in collagen content at the implant interfaces. Both strains elicited strong a tissue reaction (Fig. 4-6A-D) as well as a thick fibrous capsule on the dermal side as opposed to the muscle, which was thin (Fig. 4-6E-F & Fig. 4-7). The capsule on the dermal side was determined to be 16±4 μm for $TLR4^+$ mice and 18±2 μm for $TLR4^-$ mice while the capsule adjacent to muscle was 8±2 μm for $TLR4^+$ mice and 9±2 μm for $TLR4^-$ mice. There were no differences in the thicknesses of the fibrous capsules on either side between the two mouse strains (Fig. 4-7).

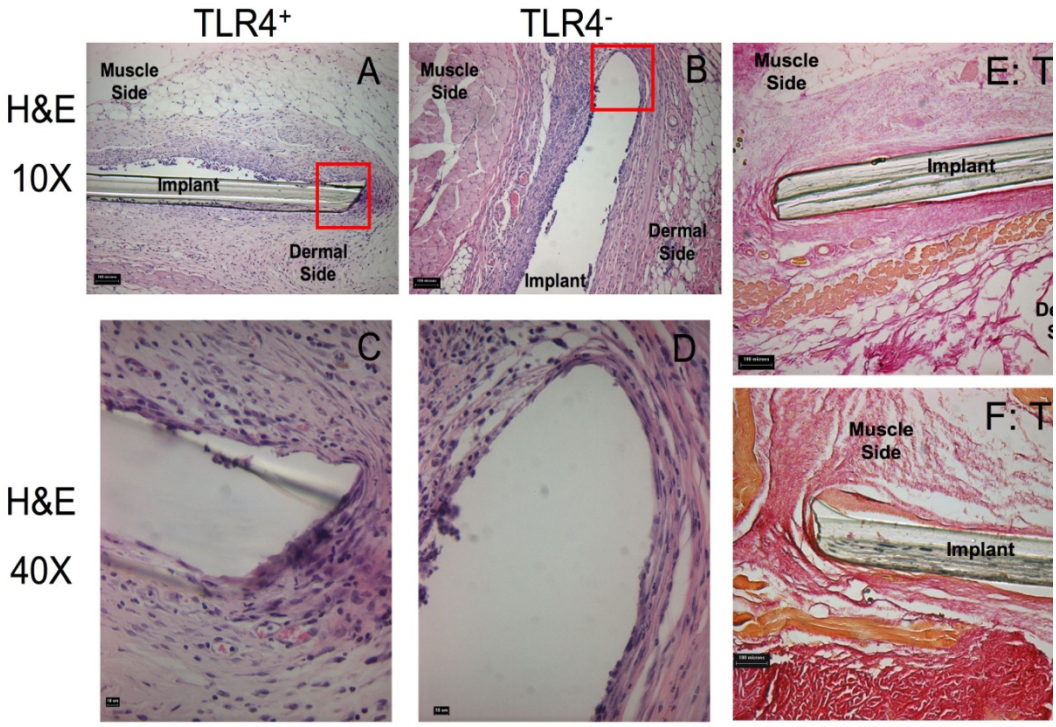

Figure 4-6: Representative H&E and Van Gieson stained tissue sections of PET discs implanted SC for 2 weeks. Shown are 10X images of representative H&E stained sections from a TLR4^{+} (A) or a TLR4^{-} (B) mouse (bar indicates 100μm) as well as 40X magnifications TLR4^{+} (C) or a TLR4^{-} (D) (bar indicates 10μm). Also shown are 10X images of Van Gieson (collagen) stained sections from a TLR4^{+} (E) or a TLR4^{-} (F) mouse (bar indicates 100μm). No noticeable differences in fibrous capsule formation were found between the two strains.

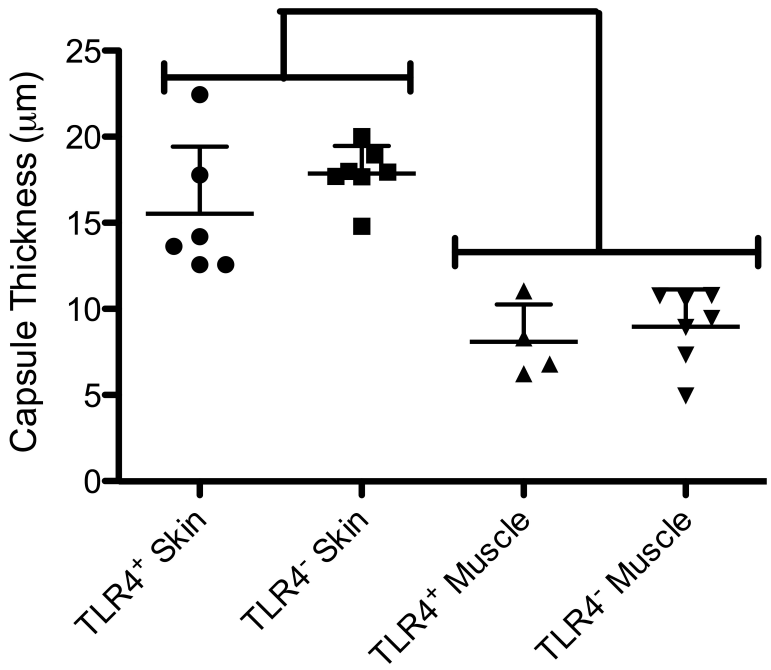

Figure 4-7: Fibrous capsule thickness surrounding PET discs implanted for 2 weeks SC in TLR4+ or TLR4- mice assessed on the skin and muscle sides. Thicknesses were averaged for the skin side or the muscle side of the implant for each animal and individual determinations are plotted as points with horizontal lines representing mean with standard deviations; n=4-7 mice per group. Brackets indicate statistical difference between skin and muscle thicknesses only ($p<0.05$).

Discussion

For the first time, this research demonstrates that TLR4 plays a role in the host response to a biomaterial *in vivo*. Specifically, mice lacking TLR4 had an altered differential leukocyte profile recovered from PET surfaces following a 16hr intraperitoneal implantation as compared to mice possessing wild-type TLR4 (Fig. 4-4B). Hence, TLR4 appears to affect the differential profile of biomaterial-adherent leukocytes while not having an effect on the differential profile of leukocytes recruited into the peritoneal cavity that were recovered in the peritoneal lavages (Fig. 4-1 and Fig. 4-2). While there were differences in the profile of adherent leukocytes following an IP implantation for 16hr, in the SC site, after 2 weeks of implantation of a PET disc, there was no dependence on TLR4 of the resultant fibrous capsule (Fig. 4-6 and Fig. 4-7). Therefore, TLR4 may be necessary for optimal activation and adhesion of early responders such as neutrophils, but the chronic inflammatory response is unaffected by the absence of TLR4.

TLRs are used by the body to respond to PAMPs and DAMPs (Takeda et al. 2003; Pandey and Agrawal 2006). TLR4 has been highlighted for recognition of numerous DAMPs (Tsan and Gao 2004) contributing to normal inflammatory homeostasis during wound repair following sterile injury by sensing 'danger'/damage (Mollen et al. 2006; Zhang and Schluesener 2006; Kaczorowski et al. 2008). Also, injury primes TLR4 for activation by increasing its responsiveness to its ligands (Paterson et al. 2003). In this study, it was hypothesized that that a lack of TLR4 in the context of a biomaterial implant would lead to lower recruitment of leukocytes following an implantation procedure as neutrophil accumulation and TNF-α production following hemorrhage-induced lung injury was lessened in TLR4-defective mice (Barsness et al. 2004). Also, macrophage-mediated inflammation following acute lung injury occurs via TLR4/TLR2 interactions with hyaluronic acid fragments (Jiang et al. 2005). Furthermore,

it was hypothesized that mice lacking TLR4 would show an altered chronic inflammatory response (fibrous capsule formation) to a biomaterial as TLR4-deficient mice exhibit a stronger wound healing response following a sterile incision (Bettinger et al. 1994).

It was hypothesized that a lack of TLR4 in the context of a biomaterial implant would lead to lower recruitment of leukocytes following an implantation procedure as neutrophil accumulation and TNF-α production following hemorrhage-induced lung injury was lessened in TLR4-defective mice (Barsness et al. 2004). Also, macrophage-mediated inflammation following acute lung injury occurs via TLR4/TLR2 interactions with hyaluronic acid fragments (Jiang et al. 2005). However, mice possessing wild-type TLR4 or lacking TLR4 elicited similar leukocyte recruitment to the peritoneal cavity following biomaterial implantation (Fig. 4-1). Sham and implant treatment groups for both strains were also similar (Fig. 4-1) implying that surgery alone may have accounted for a significant aspect of the inflammatory response. However, no statistical difference was found for leukocyte concentrations for lavages from sham or naïve groups. Others have noted that the total number of macrophages and neutrophils following a sham surgery procedure at 18hr was found to be similar to naive mice (Busuttil et al. 2004) and did not account for the majority of inflammation to PET implanted IP. However, in this study lavages (which exhibited similar total leukocyte concentrations) from sham surgeries or biomaterial implantation contained similar profiles of neutrophils (Fig. 4-2A) and monocyte/macrophages (Fig. 4-2B) for both $TLR4^+$ and $TLR4^-$ mice.

It was unanticipated that only in the presence of a biomaterial implant would TNF-α be detected IP (Fig. 4-3), furthermore in a TLR4-independent manner, wherein sham and naïve mice had essentially no detectable levels of the pro-inflammatory cytokine. Others have found that peritoneal macrophages from TLR4-defective mice produce significantly less TNF-α mRNA *in vitro* in response to hydroxyapatite particles than $TLR4^+$ mice (Grandjean-Laquerriere et al. 2007). *In vivo* a biomaterial likely

presents many other DAMPs than presented by a biomaterial *in vitro*, and these may activate cytokine production through other PRRs such as TLR2 (Tsan and Gao 2004; Yu et al. 2006; Maitra et al. 2008). In this study, however, using an IP biomaterial implantation model such a strong response to injury alone was not observed. These findings are supported by others who have shown that TLR4 does not account for inflammation (recruitment of neutrophils) IP following injection of dead cells (Chen et al. 2007). Overall, these findings imply that TLR4 was not responsible for the recruitment of leukocytes to the IP cavity or TNF-α production.

Conversely, a differential profile of adherent phagocytes on IP-implanted PET discs was observed that was TLR4-dependent. It was surprising that an altered differential leukocyte profile was observed for $TLR4^-$ mice as compared to $TLR4^+$ (Fig. 4-4B). Implants in $TLR4^-$ mice had a significantly higher fraction of recovered adherent neutrophils (~0.58) and a lower fraction of recovered adherent monocyte/macrophage (~0.36) as compared to that of $TLR4^+$ mice (Fig 4-4B). In contrast, $TLR4^+$ mice had equivalent fractions of recovered adherent neutrophils and monocyte/macrophages (about 0.47 for both) (Fig. 4-4B) from discs. This was observed even though the total number of leukocytes attaching to PET disc was equivalent between $TLR4^+$ and $TLR4^-$ mice (Fig. 4-4A), and the IP milieu surrounding the implants wherein neutrophil and monocyte/macrophage fractions were equivalent in $TLR4^+$ and $TLR4^-$ mice (Fig. 4-2A and 4-2B). In support of a role for TLR4 in controlling the adhesive leukocyte profile, another common wild-type TLR4 strain, C57BL/6, exhibited the same neutrophil and monocyte/macrophage profile at 16hr on PET to that of the $TLR4^+$ strain used here, C57BL/10 (APPENDIX 1). A limitation of this study is that trypsin removal of biomaterial-adherent leukocytes did not allow for direct cell staining due to damage of surface molecules. This prevents the use of flow cytometry to better characterize the cell population which would be beneficial in future studies.

There are two possible explanations for the observed TLR4-dependence of the adherent phagocyte profile on PET discs implanted IP. First, as neutrophils account for the majority of inflammatory cells recruited during acute inflammation in response to an implanted biomaterial within the time frame studied here, endogenous ligands present on the material surface may trigger TLR4-induced activation of neutrophils to subsequently progress the inflammatory cascade (including stimulating monocyte/macrophages). Without TLR4, neutrophils would not be fully activated by proteins on the PET surface. In fact, TLR signaling also induces up-regulation of integrin (CD11b/CD18) expression in neutrophils (Ding et al. 1999; Sabroe et al. 2003) which may even control adhesion, along with optimal activation, to a material (McNally and Anderson 1994; McNally and Anderson 2002) and explain why an apparent "delayed" inflammatory response is seen in $TLR4^-$ mice. Numerous proteins adsorb to biomaterials from physiological fluids (Wilson et al. 2005). Most of these are considered adhesive substrates, but plasmin (Ward et al. 2006), fibrinogen (Smiley et al. 2001) and fibronectin (Okamura et al. 2001) are also ligands for TLR4. Hence, such biomaterial-adsorbed proteins may concurrently induce the inflammatory cascade. Interestingly, firm adhesions via integrins also leads to optimal TLR4 signaling (Monick et al. 2002) in macrophages further supporting their activity. Therefore, upon implantation of a biomaterial, a foreign surface may offer the perfect environment for TLR4/DAMP interactions to lead to leukocyte activation: by acting as a depot for DAMPs to elicit a 'danger' response from the host as well as offering an adhesive substrate.

Lastly, TLR4-mediated phagocyte accumulation on the surface may not be a product of direct interaction with DAMPs on biomaterials but as a result from the absence of TLR4 on many other cells in $TLR4^-$ mice. TLR4 is expressed on various cell types including most immune cells, some epithelial cells and even endothelial cells (Takeda et al. 2003). The adhesion to the material surface may be indirectly controlled,

for example, by endothelial cells which may act to prime neutrophils and monocyte/macrophages for adhesion to a material surface. It is well documented that endothelial cells stimulate neutrophil activation and integrin-mediated adhesion during an inflammatory response through secretion of chemokines. It is also believed that endothelial cells sense DAMPs (such as hyaluronic acid fragments) through TLR4 which induce chemokine (IL-8) secretion (Taylor et al. 2004). Therefore, endogenously activated endothelial cells may ready neutrophils for adhesion through paracrine chemokine interactions.

The lack of TLR4-dependence on the fibrous capsule formation at 2 weeks to PET implanted subcutaneously was anticipated (Fig. 4-6 and Fig. 4-7) as other TLRs (such as TLR2) may compensate for DAMP interactions. A significant decrease in tissue reaction to an implanted biomaterial would be anticipated in mice which lack MyD88 (a common adapter molecule required for nearly all TLR family member signaling, with the exception of TLR3). MyD88$^{-/-}$ mice had an almost completely abrogated inflammatory response to DAMP-mediated inflammation (Chen et al. 2007). Interestingly, IL-1R (which shares identical signaling pathway with TLRs) was, and has been shown by others, linked to these effects. Specifically, intracellular inflammasome activation of IL-1/IL-1R signaling may be the cause for 'danger'-induced effects including the adjuvanticity of alum (Li et al. 2007) and other biomaterial particulates (Sharp et al. 2009) used for vaccines. However, the potential for a flat and large surface such as the PET disc implanted herein to interact intracellularly with inflammasome components to induce IL-1 activation in the absence of internalization of the material due to size remains to be determined but seems unlikely.

In conclusion, TLR4 plays a role in determining the adhesive leukocyte profile on biomaterials implanted intraperitoneally during the acute inflammatory response. This may be mediated by a direct interaction with DAMPs adsorbed on the material surface or

indirectly affected by other cell types, which prime leukocytes for adhesion. TLR4 blocking may be beneficial if trying to direct an acute host response that minimizes monocyte/macrophage adhesion to a biomaterial. Fibrous encapsulation following subcutaneous implantation of a biomaterial propagates normally in the absence of TLR4 indicating other compensatory receptors for activation and support of the foreign body tissue response. Therefore, the window of activity for TLR4 in the host response to a biomaterial is only in the initial recognition of the foreign entity.

CHAPTER 5

ROLE OF 'DANGER SIGNAL'-TLR4 INTERACTIONS IN THE DENDRITIC CELL RESPONSE TO BIOMATERIALS

Introduction

Dendritic cells act as a bridge to link the body's innate immune response to its antigen-specific adaptive immune response. Similar to other leukocytes, they have evolved to respond directly to pathogen invasion by recognizing pathogen-associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs) (van Vliet et al. 2007). The recognition process by PRRs induces internal signaling which culminates in the production of inflammatory cytokines, up-regulation of co-stimulatory molecules (CD80, CD86) and may also induce migration of DCs to the lymph nodes for the induction of an adaptive immune response (Banchereau and Steinman 1998). However, there is building evidence that many PRRs not only respond to PAMPs but also recognize endogenous 'danger signals'.

'Danger signal' molecules (or DAMPs) include fragmented/denatured ECM components or molecules normally sequestered in cellular cytoplasm which upon tissue damage or cell necrosis become visible to the environment (Skoberne et al. 2004). Intracellular molecules from stressed/dying cells can act as adjuvants and increase antibody production toward a model antigen *in vivo* (Gallucci et al. 1999). Previously, we have seen an adjuvant effect *in vivo* to ovalbumin co-delivery with biomaterial PLGA scaffolds or MPs but not with agarose scaffolds (Matzelle and Babensee 2004; Bennewitz and Babensee 2005; Norton et al. 2010), which are common materials used in tissue engineering devices. It is hypothesized that this response is due to DC interaction with biomaterials as materials such as PLGA or chitosan induce DC maturation while materials such as agarose or hyaluronic acid limit or do not significantly

affect DC maturation *in vitro* (Yoshida and Babensee 2004; Babensee and Paranjpe 2005; Yoshida and Babensee 2006). The goal of the research presented here is to examine whether biomaterial-induced DC maturation is mediated by DC interaction with endogenous 'danger signal' molecules present on biomaterials as part of the adsorbed protein layer. Elucidating the mechanism behind the DC response to biomaterials may allow for improved biomaterial selection for tissue engineering scaffold in order to inhibit or minimize the biomaterial-induced adjuvant effect. Conversely, biomaterial section can be support for vaccine delivery systems to enhance immune responses through an adjuvant effect.

PRRs present on the surface of DCs which may be used to recognize both PAMPs and DAMPs include (but are not limited to) C-type lectin receptors (CLR), integrins / complement receptors (CR), scavenger receptors (SR) and Toll-like receptors (TLR) (Lee and Kim 2007) (see LITERATURE REVIEW). CLRs (such as DC-SIGN) recognize carbohydrate structures derived from bacteria (such as mannose) as well as carbohydrate structures present on the surface of apoptotic cells (Figdor et al. 2002; Gijzen et al. 2006; van Vliet et al. 2006). Specific β_2 integrins ($\alpha_M\beta_2$, $\alpha_X\beta_2$, also known as CR3 and CR4, respectively) on DCs recognize C3b, which has opsonized bacteria for clearance (Gasque 2004; Bajtay et al. 2006) but can also bind fibrinogen, a host protein found in high local concentrations following tissue damage (Hu et al. 2001; Thacker and Retzinger 2008). The SR family can recognize numerous bacteria-derived polyanionic ligands (Peiser et al. 2002) such as lipoteichoic acid but may also bind intracellular chaperone proteins HSPgp96, HSP70 and HSP90 (Facciponte et al. 2005). Lastly, the most well characterized PRR family is the TLR family, which consists of at least 12 members in mammals which are believed to recognize ligands through leucine-rich repeats present in their horseshoe-shaped ectodomain (Akira et al. 2006; Park et al. 2009). TLR members, in particular TLR2 and TLR4, have been found to bind a wide

variety of PAMPs and 'danger signal' molecules (Tsan and Gao 2004; Rock and Kono 2008).

Of the TLR family members, TLR4 is the most characterized including understanding its precise intracellular signaling pathway (Kawai and Akira 2006) (see Fig. 3-2) which concludes in the activation of two key transcription factors nuclear factor: (NF)-κB and AP-1 (see LITERATURE REVIEW). The prototypical ligand for TLR4 is LPS, a component of Gram-negative bacteria (Hoshino et al. 1999; Guha and Mackman 2001); however, TLR4 has been linked to the recognition of a wide variety of endogenous molecules including fibrinogen (Smiley et al. 2001), fibronectin (Okamura et al. 2001), hyaluronic acid (Taylor et al. 2004; Jiang et al. 2005) and HMGB1 (Yu et al. 2006) as well as others (see LITERATURE REVIEW). Recently, TLR4 was found to control the adherent leukocyte profile present on a biomaterial implant (Rogers and Babensee 2010) as well as being involved in macrophage recognition and inflammatory response to hydroxyapatite particles (Grandjean-Laquerriere et al. 2007) and alginate (Iwamoto et al. 2005). Thus, TLR4 has been shown to be participating, in part, in the induction of an inflammatory response to biomaterials.

The overall goal of the work presented in CHAPTER 5 was to determine whether TLR4/'danger signal' interactions occur on the surface of biomaterials and whether these interactions account, in part, for biomaterial-induced DC maturation. Specifically, TLR4-expressing HEK293 cells were utilized to isolate the role of potential TLR4/'danger signal'-induced signaling via NF-κB and AP-1 activation. Bone marrow derived DCs (BMDC) were derived from $TLR4^+$ or $TLR4^-$ mice and treated with PLGA films or MPs to assess the contribution of TLR4 to DC maturation in response to this biomaterial. Lastly, TLR-related gene expression patterns in PLGA or agarose treated human monocyte-derived DCs were analyzed in order to determine other potential TLRs involved in the response to biomaterials. DC gene expression patterns in response to biomaterials were

also compared to LPS treatments to ascertain if the response to biomaterials is similar or different to that of PAMP stimulation.

Materials and Methods

Biomaterial Preparation

A solution of 10% w/v PLGA (mole ratio 75:25, inherent viscosity 0.70 dL/g in trichloromethane; Durect/Lactel Absorbable Polymers, Birmingham, AL) was prepared in 20mL dichloromethane overnight in sterile polypropylene tubes (Yoshida and Babensee 2004). To form films, this solution was gently poured into a cleaned 100mm Teflon dish and allowed to sit for two days in fume hood until DCM had evaporated. PLGA films were either cut for use in 100mm dishes, or punched out with arch punches for 24 well plates (9/16'') or 6 well plates (5/4'') and rinsed for 1h with sterile water. Films were allowed to dry in biosafety cabinet, sterilized with UV (30min each side) and rinsed with endotoxin free water immediately prior to use. Films were placed in bottom of wells for treatment of cells as described below.

PLGA MPs were prepared by a single emulsion solvent evaporation technique (Yoshida and Babensee 2004). Briefly, a 2.5%(w/v) solution of PLGA in dichloromethane (Sigma) was prepared by with dissolution overnight and homogenized in 0.3% poly(vinyl alcohol) (Sigma). Solvent was evaporated overnight, and MPs were collected and rinsed several times in endotoxin-free water and exposed to UV (1hr) immediately prior to DC treatment at a 5:1 MP:cell ratio. Size distribution was determined using Coulter Counter and found to be of a peak size of 3.8μm.

Three percent (w/v) agarose (Type V) (Sigma, St. Louis, MO) films were prepared by microwaving agarose/water suspension for 30s. This solution was then cast in 6 well plates and allowed to cool at 4^{o}C for 30min. The films were brought to room temperature for at least 1hr before use in cell culture.

Endotoxin content was evaluated using Limulus Amebocyte Lysate (LAL) assay (QCL-1000, Lonza, Basel, Switzerland) with detected levels of approximately 0.1 EU/mL

for all biomaterials used and tested below the FDA endotoxin limit for medical devices (0.5 EU/mL).

TLR4-expressing HEK293 Cell Examination of Biomaterial Effects

Stable Silencing of HEK293 Cells

HEK293 cells which stably over-expressed TLR4, CD14 and MD2 (InvivoGen) (TLR4-expressing HEK293 cells) were used for biomaterial and LPS treatments. A stably silenced transformation of TLR4-expressing HEK293 cells was created using an shRNA-expressing plasmid, which was directed toward MyD88 for knock-down (InvivoGen). The plasmid was pre-complexed with a lipid-based transfection delivery agent and also induced the production of green fluorescent protein (GFP) for determining transfection efficiencies and Zeocin resistance for stable selection. TLR4-expressing HEK293 cells were transfected according to manufacturer's instructions in 12 well plates (Corning) at $2*10^5$ cells per well. As another negative control, wild-type HEK293 cells (293-null, InvivoGen), which did not over-express any surface receptors, were also utilized.

Cells were cultured in T-75s (BD Falcon) prior to treatment and collected using Trypsin/EDTA (0.25%) (Sigma) followed by addition of cell culture media (DMEM with high glucose, pyridoxine hydrochloride, without L-glutamine or sodium pyruvate, (Invitrogen) supplemented with 10% heat-inactivated FBS (Cellgro) containing selective antibiotic Blasiticidin S (InvivoGen) (10µg/mL) for 293-null, Blasticidin S + Hygrogold™ (InvivoGen) (50µg/mL) for TLR4-expressing HEK293 cells, or Blasticidin S + Hygrogold™ + Zeocin (InvivoGen) (100µg/mL) for MyD88-silenced TLR4-expressing HEK293 cells) to inactivate trypsin.

Transfected or non-transfected TLR4-expressing HEK293 cells were stably selected in 6 well plates using Zeocin (InvivoGen) (100 µg/mL) over 10-14 days following transfection, and selection was determined via flow cytometry for GFP expression.

Biomaterial Treatment of HEK293 Cells

HEK293 cells (TLR4-expressing HEK293 cells, MyD88-silenced TLR4-expressing HEK293 cells, or wild-type HEK293 cells) were cultured in T-75 as described and trypsinized for collection and subsequent treatment. Cells were then resuspended at $8*10^5$/mL and plated in 100mm dishes (Primaria, BD Falcon) with 25mL per dish. Cells were left untreated, treated with LPS (Sigma, *E. coli* 055:B5) at 1µg/mL or plated on top of either PLGA films placed in dishes or agarose films prepared directly in dish for use. Cells were placed in 37^{o}C incubator for 5 or 24hr. Cytosolic and nuclear extracts were prepared as described below. Supernatants were also saved and stored at -20^{o}C for chemokine secretion analysis.

Cytosolic and Nuclear Extraction Procedures

To prepare either cytosolic or nuclear extract from HEK293 cells, cells were lysed using a protocol which specifically prepares both cytosolic and nuclear fractions of cell lysates (Clontech, Mountain View, CA). Ice-cold PBS was added to cells following treatment, and they were collected using cell scrapers (BD Bioscience). Cells were centrifuged at 450g for 5min and washed with cold PBS once again. Cell suspensions were counted using Coulter Counter. Cell pellet volume was estimated and the amount was be used for subsequent resuspension volumes. Lysis buffer (Clontech) (5x the cell pellet volume) was added to the cell pellet and incubated on ice for 15min. Cells were then centrifuged at 450g for 5min, and the supernatant carefully discarded. The cell pellet was then resuspended in lysis buffer (volume equal to 2x the original cell pellet)

and suspension was passed through a 28g needle 10 times to disrupt cell membranes. Lysed suspension was centrifuged at 10,500g for 20min, and the supernatant (cytosolic extract) collected and stored at -80^{o}C. Nuclear extraction buffer (Clontech) was then added at 2/3 the volume of the original cell pellet to the remaining pellet, and the nuclear membranes disrupted with a 28g needle as before. Suspension was then shaken gently (1000rpm) for 30 min at 4^{o}C using Thermomixer (Eppendorf, New York, NY). Suspension was centrifuged at 20,500g for 5min, and the supernatant (nuclear lysate) collected and stored in a fresh pre-chilled low retention microcentrifuge tube. Bicinchononic acid (BCA) assay (Sigma) was used to determine the protein concentration of both nuclear and cytosolic extracts.

Western Blot to Verify MyD88 Knock-Down in TLR4-expressing HEK293 Cells

To assure MyD88-knock down in the transformed TLR4-expressing HEK293 cells, western blotting of cytosolic extract of stably selected cells was used to determine the expression of MyD88. Cytosolic extracts were prepared as described above and probed for MyD88 expression using common SDS-gel electrophoresis with subsequent immunoblotting techniques. SDS-PAGE was performed following manufacturer's protocol (Invitrogen). A normalized amount of 20µg of protein was loaded per well for gel electrophoresis. NuPAGE® LDS sample buffer (4x), NuPAGE® Reducing agent (10x) (both from Invitrogen), and 20 µg of protein were added to a low-retention microcentrifuge tube followed by addition of deionized water for a total of 10µL solution per sample. The samples were then each heated to 70^{o}C for 10min in a heat block and immediately used. MyD88 was probed using a low-molecular weight NuPAGE® 4-12% Bis-Tris Gel (Invitrogen), which has a resolution between 10-200 kDa. Prepared samples and protein standard (Precision Plus Protein Standards, Kaleidoscope, Bio-Rad, Hercules, CA) were loaded simultaneously into XCell SureLock™ Mini-Cell apparatus

(Invitrogen) after filling the upper chamber with 200mL of MOPS SDS Running Buffer (Invitrogen). 200V (constant) was applied across the gel using a Power Source (250V) (VWR, West Chester, PA) for 45-50min.

Following completion of electrophoresis, proteins were transferred to nitrocellulose membranes (Invitrogen) using the XCell SureLock™ apparatus. Gels were removed from cassettes and immediately placed on top of filter paper, which was placed on top of two blotting pads. A nitrocellulose membrane was placed carefully on top of the gel, and the gel was kept wet with NuPAGE® transfer buffer (Invitrogen). A separate filter paper was placed on top of the membrane followed by two blotting pads. The entire "sandwich" was loaded into the cathode core of the blot module and placed into the apparatus. The upper chamber was then filled with transfer buffer while the lower chamber filled with deionized water. Transfer took place at 30V for 1hr. The membrane was carefully removed and blocked by gentle shaking in 0.1% PBS/Tween 20 (Sigma) with 5% (w/v) milk for 1hr. Next, membranes were incubated with appropriate dilutions of rabbit anti-human MyD88 (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-β actin (run in separate but simultaneously prepared membranes) in 0.1% PBS/Tween 20 (Sigma) with 5% (w/v) milk overnight at 4^{o}C. Membranes were then gently washed 3 times for 5min in 0.1% PBS/Tween 20 followed by addition of diluted secondary antibodies conjugated to alkaline phosphatase (AP) (Southern Biotech, Birmingham, AL). Membranes were lastly washed 3 times for 5min as before following by addition of AP substrate (Bio-Rad, Hercules, CA) directly to membrane. Protein bands were detected using Hyperfilm ECL (GE Healthcare, Piscataway, NJ) placed in cassette with membrane and subsequently developed.

Caspase Analysis

Cytosolic extracts were harvested and examined for early markers of apoptosis

(activated caspase 8 and caspase 3) by a fluorometric technique (BioSource). Briefly, equivalent amounts of cell lysates (50μg per 50μL) were added to a reaction buffer (50μL) containing 10mM DTT. Next, 5μL of synthetic peptide DEVD-AFC (7-amino-4-trifluoromethyl coumarin) (for caspase 3 analysis) substrate or synthetic peptide (IETD)-AFC (for caspase 8 analysis) substrate was added to cytosolic extracts. Samples were incubated for 30min at room temperature and read at 520nm following an excitation at 492nm. Relative levels of activated caspase were compared to untreated cell lysates for each cell line.

Transcription Factor Activation of Biomaterial-Treated HEK293 Cells

Transcription factor activation (NF-κB and AP-1) was determined using an ELISA based technique in which DNA-oligomers are pre-coated to the bottom of wells and bind only activated NF-κB (Clontech) or AP-1 (TransAM™ AP-1 Family kit, Active Motif, Carlsbad, CA). As determined by BCA assay, 20μg of nuclear extracts from treated cells was equivalently loaded onto plates. For NF-κB analysis, loaded extracts were incubated for 60min. Plates were washed and incubated with primary antibodies (in separate wells) recognizing each of the NF-κB family members (p50, p65, p52, RelB, and cRel) for 60min. After washing again, a secondary antibody was added and tetramethylbenzidine (TMB) substrate added and colorimetric development determined at 655nm. For AP-1 analysis, similar techniques were used except AP-1 primary antibodies added will recognize cFos, FosB, Fra-1, Fra-2, cJun, JunB, and JunD. Secondary antibody was incubated following addition of developing agent and stop solution (acetic acid) and color development read at 450nm with a reference of 655nm.

Chemokine/Cytokine Detection in Supernatants

IL-8 secretion (normalized to cell counts collected using Coulter Counter) was determined using sandwich ELISA-based technique. Briefly, supernatants were added to human IL-8 ELISA kit (R&D Systems) 96-well plates and incubated for 60min. After washing, plates IL-8 conjugate was added for an additional 60min. Lastly, developing solution (TMB) was added followed by stop solution. Optical densities were determined at 655nm. For biomaterial-treated BMDCs or human DCs, TNF-α concentration in supernatants was also determined via similar ELISA kits (R&D Systems).

Bone Marrow Derived Dendritic Cell Examination of Biomaterial Effects

Mouse Strains

Male mice (6 weeks in age) of three strains were used as the source of bone marrow (BM): C57BL/10 and C57BL/10ScSn, both $TLR4^+$ as well as the TLR4-defecient strain, C57BL/10ScN ($TLR4^-$) (Poltorak et al. 1998) (all from The Jackson Laboratory, Bar Harbor, ME).

BMDC Culture

BMDCs were prepared from $TLR4^+$ or $TLR4^-$ mice concurrently on the same day. Mice were sacrificed at Emory University Whitehead biomedical research facility in accordance with an IACUC approved procedure (IACUC protocol #044-2006). This method is based on a previously established method with modifications (Inaba et al. 1992; Yoshida et al. 2007). Femurs and tibias were collected and BM was harvested by flushing 5mL of mouse DC media [Dulbecco's Modified Eagle Medium (DMEM) with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 1% (v/v) sodium pyruvate, 1% (v/v) non-essential amino acids, 1% (v/v) HEPES (all from Cellgro, Manassas, VA), 0.1% (v/v) 2-mercaptoethanol (Invitrogen, Carlsbad, CA), and 1% (v/v) penicillin-streptomycin (Cellgro)] using a 26g needle and collected in a 60mm tissue culture dishes. BM was

passed through a 70μm filter. Red blood cells (RBC) were lysed using a sterile-filtered ammonium chloride solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, all from Sigma, St. Louis, MO) and rinsed twice with 2% FBS/Dulbecco's-PBS (D-PBS, Invitrogen). Cells were then counted and resuspended at a concentration of $1.5*10^6$ cells/mL in mouse DC media and plated in 6 well plates (Costar, Corning, Corning, NY) with 3 mL/well and supplemented with murine GM-CSF (BD Biosciences, San Diego, CA) and murine IL-4 (Sigma) both at concentrations of 20ng/mL at 37°C and 5% CO_2. On day 3 of culture, half of media from each well was carefully collected and re-supplemented with fresh cytokine containing media. On day 6, non/loosely-adherent cells were collected by gentle swirling and rinsed twice with 2%FBS/D-PBS with 2mM EDTA. $CD11c^+$ cells were isolated using CD11c-magnetic beads (Miltenyi Biotec, Auburn, CA) and LS columns (Miltenyi Biotec) in association with a midi-MACS magnet (Miltenyi Biotec) following manufacturer's instructions. Collected $CD11c^+$ cells were resuspended at a concentration of 10^6 cells/mL in mouse DC media containing cytokines and used as immature DC (or "iDC") for remaining experiments performed in 24 well plates (Costar, Corning) with 1 mL of DC suspension per well.

Biomaterial Treatment of BMDCs

On Day 6 of BMDC culture following CD11c-isolation, non/loosely-adherent iDCs from either $TLR4^+$ or $TLR4^-$mice were collected and resuspended at 10^6 cells/mL in mouse DC media containing cytokines and used as iDC for remaining experiments performed in 24 well plates (Costar, Corning) with 1mL of DC suspension per well. iDC were either left untreated, treated with ultrapure-LPS (InvivoGen) (1μg/mL), or treated with PLGA films or MPs for 24h at 37°C. Following treatment, non/loosely-adherent DCs were collected, rinsed with FACS buffer (96% (v/v) Hank's HEPES Buffer (120mM NaCl, 10mM KCl, 10mM $MgCl_2$, 10mM glucose, and 30mM HEPES. All chemicals from

Sigma), 1% (v/v) Human serum albumin (HSA) (EMD Biosciences, La Jolla, CA), and 1.5mM $CaCl_2$) and supernatants collected for cytokine analysis. Cells were stained with antibodies recognizing murine CD11c (BD Pharmingen), I-A^b (BD Pharmingen), CD80 (BioLegend, San Diego, CA), CD86 (BioLegend) or recombinant Annexin-V (BD) for 1hr at 37^{o}C and run on flow cytometer (BD LSR II, BD Biosciences). Immediately prior to flow cytometry, cells were also stained with propidium iodide to assess cellular necrosis.

Human Monocyte-derived DC Examination

Toll-like Receptor Gene Expression in Human DCs

Human peripheral blood was drawn at Georgia Institute of Technology Student Health Center in the phlebotomy laboratory following approved IRB protocol (H05012) procedures. Human DCs were cultured from peripheral blood mononuclear cells (PBMCs) as described previously (Romani et al. 1996) with some modifications. PBMCs were isolated from buffy coats following centrifugation at 400g for 30m using lymphocyte separation media (Cellgro). Red blood cells were lysed using sterile Lysis Buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA). After washing cells twice with D-PBS (Invitrogen, pH 7.4), they were resuspended at a concentration of $5*10^6$/mL in human DC media (RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated (57^{o}C, 30 min) FBS (Cellgro) and 1% (v/v) penicillin/streptomycin (Cellgro). PBMCs were isolated based on adhesion to 100mm tissue culture plate (Primaria, BD Falcon) after 2 hr incubation at 37^{o}C. Plates were washed 3 times with DC media to remove non-adherent cells, and resulting adherent cell population was reconditioned with DC media (10mL/plate) containing human GM-CSF (1000U/mL) and human IL-4 (800U/mL) (both from Peprotech, Rocky Hill, NJ) for 5 days at 37^{o}C, 5% CO_2. Loosely-adherent cells were dislodged by gentle swirling and collected carefully with pipettes and transferred to sterile 50mL centrifuge tubes. Cells collected were termed immature DC (iDC) and were

used directly for culture on biomaterials after being pelleted and resuspended in cytokine containing DC media ($5*10^5$/mL).

Human monocyte-derived DCs were then treated as iDC, mDC (1μg/mL ultrapure-LPS, InivoGen) or treated with PLGA or agarose films in 6 well plates for 24hr. Loosely-adherent DCs were collected by pipetting and pooled with adherent DCs which were collected using cell dissociation solution (Sigma). These pooled cells were then collected at 1100rpm for 10min. To investigate the gene expression in treated DCs alone, DCs were purified by positive selection using DC-SIGN (CD209) microbeads (Miltenyi Biotec) with MACS. Cells were resuspended in 120μL of ice-cold MACS buffer followed by 40μL of human CD209 microbeads (Miltenyi Biotec) with 40μL of Fc-receptor blocking buffer (Miltenyi Biotec) and incubated at 4°C for 15min. Cells were rinsed with 2mL of MACS buffer, collected at 400g for 10min and resuspended in 500μL of MACS buffer and passed through an LS column (Miltenyi Biotec) for purification. The efficiency of this purification procedure was confirmed using flow cytometry to assure purification of DC which was routinely >95% DC-SIGN$^+$ (APPENDIX 2).

Total RNA extracts were prepared from each treatment using RNeasy Mini kit (Qiagen). Briefly, cells were lysed and homogenized by passing cell pellets (resuspended in 400μL of Buffer RLT) through 21-gauge needles fitted to an RNase-free syringe 10x until obtaining a homogenous lysate. An equal volume of 70% ethanol (Sigma) was added to the lysate, mixed by gentle pipetting and transferred to an RNeasy spin column (Qiagen) placed in a 2mL collection tube for centrifugation at 9000g for 15s. After discarding the flow-through, this process was repeated using 700μL of Buffer RW1 and followed by Buffer RPE twice. Lastly, the spin column was placed in a new 1.5mL tube and 50μL of RNase-free water spun through column as before for 1 min to elute RNA. Concentration of RNA was determined by A_{260} method while RNA purity was determined using A_{260}:A_{280} ratio (minimum value needed greater than 2) using a

NanoDrop 1000 (Thermo Scientific). RNA was stored at -80°C. cDNA was then prepared according to manufacturer's guidelines using RT^2 First Strand Kit (SuperArray Bioscience, Frederick, MD). Equivalent amounts of total RNA extracted for each treatment and trial (1µg per 96-well array) was incubated with GE Buffer and RNase-free water for 5 min at 42°C to remove genomic DNA contamination and chilled on ice for one minute. A reverse transcription cocktail containing BC3, P2, RE3 buffers (all from First Strand kit, proprietary buffers) was prepared in RNase-free water. First strand cDNA synthesis reaction was performed according to manufacturer's protocol (incubation at 42°C for 15 min followed by 95°C for 5 min), and cDNA was immediately loaded across 96 well plate for Human Toll-like Receptor Signaling Pathway RT^2 Profiler™ PCR Array System (SuperArray Bioscience, Frederick, MD). This system allows for the evaluation of mRNA expression of 84 genes associated with TLR signaling pathway including all human TLRs, a variety of pro-inflammatory cytokines (IL-1, TNF-α), kinases such as JNK and IRAK4 and the common TLR adapter molecule MyD88. Each well of the PCR 96-well plate contains a unique primer set for each gene allowing for the individual assessment using quantitative real-time PCR techniques (SYBR-green detection).

Each of the four treatments were analyzed on a separate 96-well plate array and normalized to averaged β-actin and β_2-microglobulin [B2M] expression which was not affected by treatment. For each treatment, 1275µL of 2X SuperArray RT^2 qPCR Master Mix, 102µL of cDNA and 1173µL of ddH_2O was added together into a sterile reservoir, mixed gently and distributed (25µL per well) using a multi-channel pipette across the plate assuring equal distribution. Using a StepOne Plus qPCR System (Applied Biosystems, Foster City, CA), samples were kept for 10min at 95°C to activate DNA polymerase followed by 40 cycles of alternating 95°C for 15s and 60°C for 1min. SYBR green fluorescence was detected and used to calculate the threshold cycle (C_t) for each

well after manually setting the baseline and threshold values (same settings used across all trials and treatments). C_t values were normalized to averaged C_t values of β-actin and B2M (ΔC_t) for inter-assay comparisons and up or down-regulation of gene expression was determined by comparing ΔC_t values from treated DCs' for a specific gene with that of iDCs' ($\Delta\Delta C_t$ method). For statistical analysis of each gene, ANOVA (general linear model) was performed using Minitab to assess differences between $-\Delta C_t$ values of each treatment when donors were nested within treatment (n=3). Hierarchical 2D cluster analysis was performed using Matlab *clustergram* function assuming Pearson distribution function.

Immunoblotting for Activated Transcription Factors in Biomaterial-Treated DCs

Human monocyte-derived DCs were treated as described in text, purified, pooled and rinsed twice in ice-cold PBS. RIPA buffer (Pierce) was added and used to resuspend cell pellet at a ratio of 1mL per $5*10^6$ cells, which was determined via Coulter Counter cell counts. This suspension was kept on ice and shaken for 15m. The resulting mixture was then centrifuged at 14,000g for 15m. Supernatants were collected and stored at -80^oC for western blot analysis. Western blot procedure was performed as described, except with secondary antibodies conjugated to IRDye (Li-Cor, Omaha, NE). Membranes were then imaged using Odyssey Imaging system (Li-Cor). Primary antibodies used were as follows: anti-human total Elk-1 and anti-pS383 Elk-1 (Cell Signaling Technology, Danvers, MA); anti-human total NFkB1 and anti-pS939 NFkB1 (Cell Signaling Technology); anti-human total cFos and anti-pT235 cFOS (Abcam, Cambridge, MA). An undergraduate student Ravi Patel was responsible for the completion of several of these western blots. DC lysates from three independent trials were simultaneously assessed.

Statistical Analysis

Statistical analysis was performed on all treatment-control ratios by comparing treatment values to (1) using a student t-test (using Excel software). To compare across treatments, a one-way ANOVA analysis will be performed using Minitab Software (Version 14, State Collage, PA).

Results

TLR4-Signaling Investigated Using HEK293 Cells

To isolate the role of TLR4-induced signaling following biomaterial or an LPS-positive control treatment, a stably transfected model cell line was used. HEK293 cells stably transfected with TLR4, MD2 and CD14 (*InvivoGen*) were treated with LPS, PLGA films or agarose films and analyzed for NF-κB activation and IL-8 secretion. Higher levels of active family members (p50, p65, cRel, p52) of NF-κB were found in nuclear extracts at both 5 and 24hr in LPS-treated TLR4-expressing HEK293 cells (Fig. 5-1). PLGA or agarose films, however, did not induce any significant alteration in NF-κB activation across the family members investigated (Fig. 5-1). Accordingly, PLGA or agarose films did not induce IL-8 secretion while LPS induced significantly higher levels of IL-8 secretion (Fig. 5-2).

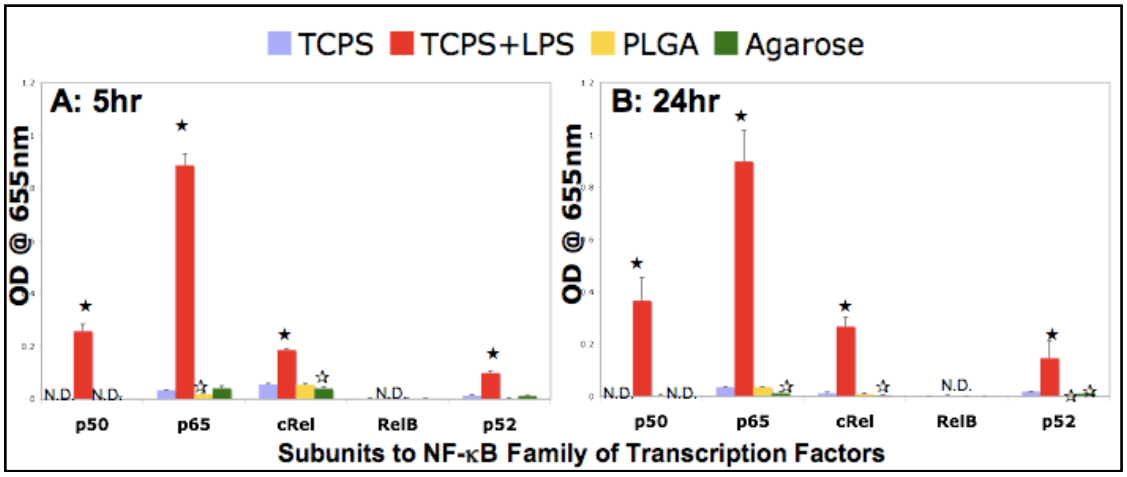

Figure 5-1: Activation of NF-κB family members (p50, p65, cRel, RelB, p52) from nuclear extracts prepared of LPS, PLGA or agarose treated TLR4-expressing HEK293 cells. 5hr (A) and 24hr (B) time points. ★, greater than TCPS, $p \leq 0.05$; ☆, less than TCPS, $p \leq 0.05$. N.D.= not detectable in assay. Representative results of three independent experiments are shown.

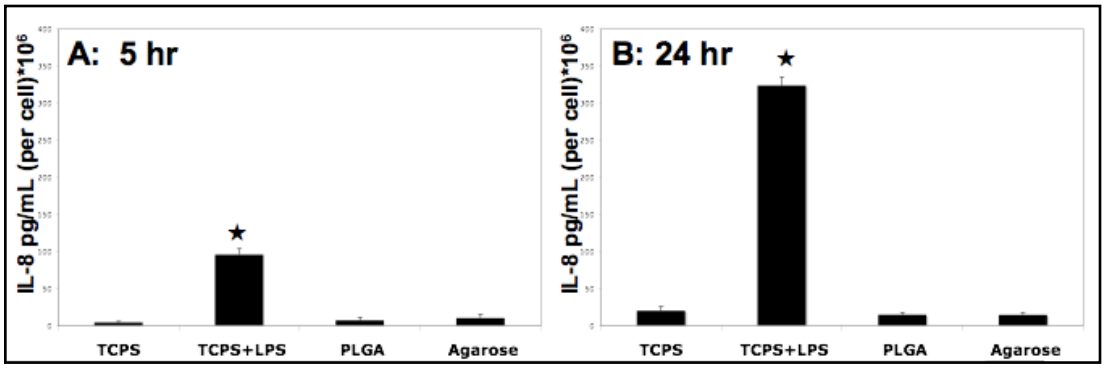

Figure 5-2: ELISA determined concentrations from cleared supernatants for IL-8 from LPS, PLGA or agarose treated TLR4-expressing HEK293 cells. 5hr (A) and 24hr (B) time points. ★ indicates greater than TCPS, $p \leq 0.05$. Concentrations were normalized by collected cell counts at each time point for each experiment. Representative results of three independent experiments are shown.

Next, since the TLR4-induced signaling pathway simultaneously activates the AP-1 transcription factor family through the MAPK pathway, AP-1 activation was similarly assessed in TLR4-expressing HEK293 cells following treatment with LPS, PLGA or agarose films for 5 or 2hr. It was found that most AP-1 family members (cFos, FosB, Fra-1, cJun and JunD) were activated by all treatments at 24hr. (Fig. 5-3B).

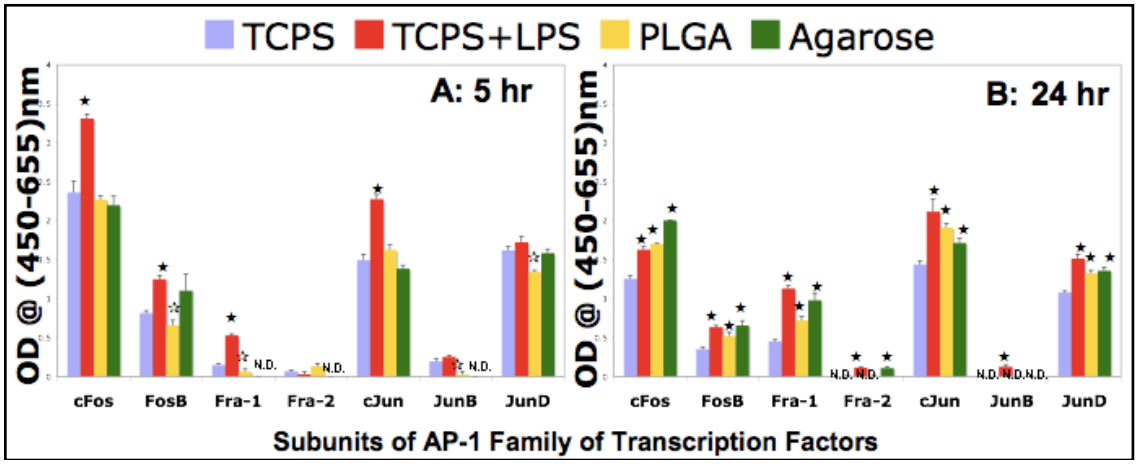

Figure 5-3: Activation of AP-1 family members (cFos, FosB, Fra-1, Fra-2, cJun, JunB, JunD) from nuclear extracts of TLR4-expressing HEK293 cells analyzed by ELISA (*ActiveMotif*). 5hr (A) and 24hr (B) time points. ★ greater than TCPS, $p \leq 0.05$; ☆, less than TCPS, $p \leq 0.05$. N.D.= not detectable in assay. Representative results of three independent experiments are shown.

In order to investigate if TLR4-signaling pathways were being utilized in the differential transcription factor response (LPS through NF-κB and AP-1 while biomaterials through only AP-1), stable MyD88 knockdowns of TLR4-expressing HEK293 cells were produced using a shRNA-expressing plasmid directed against MyD88 (Fig. 5-4). TLR4-expressing 293 cells were assessed for GFP expression via flow cytometry. It was found that the stably selected transfected TLR4-expressing HEK293 cells were 98% GFP^+ (Fig. 5-4B) confirming plasmid uptake, expression and purity of the newly transformed cell population. Effective MyD88 knockdown was determined using western blotting techniques of cytosolic extracts (Fig. 5-5). MyD88-silenced and non-transformed TLR4-expressing HEK293 cells were then treated with LPS, PLGA or agarose films for 24hr. However, stably-silenced MyD88 TLR4-expessing HEK293 cells were still able to respond to LPS as seen with increased levels of NF-κB activation (p65 subunit only) in comparison to TCPS controls, and the response was similar to non-transformed TLR4-expressing HEK293 cells (Fig. 5-6).

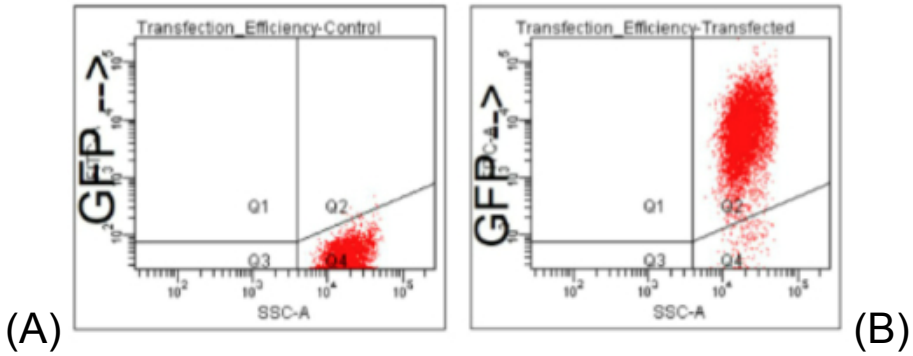

Figure 5-4: MyD88 Plasmid Delivery and Stable Selection in TLR4-expression HEK293 Cells. Confirmed via flow cytometry. Non-transfected (A) and transfected TLR4-expressing HEK293 cells (B) were assessed for GFP expression. Cell population was gated and dot plots shown are GFP vs SSC.

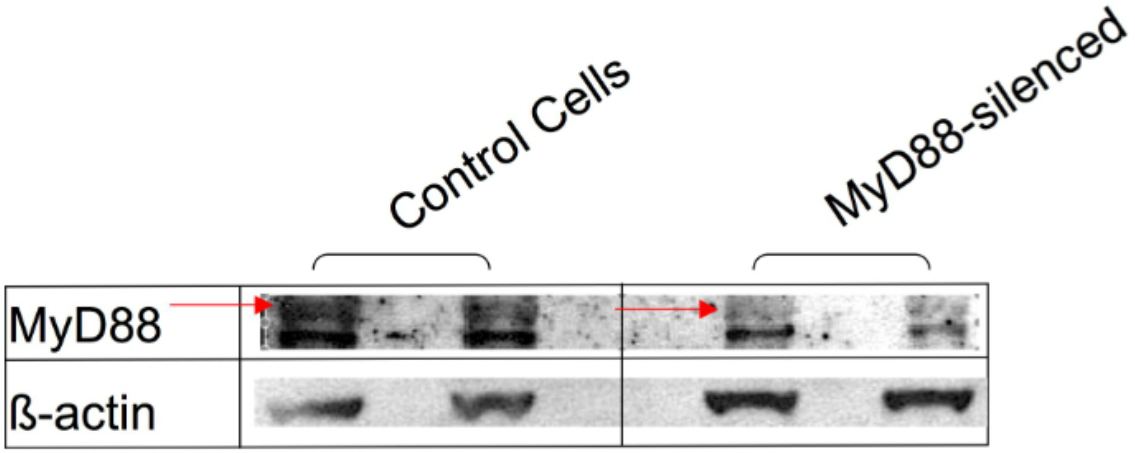

Figure 5-5: MyD88 protein expression in TLR4-expressing HEK293 cells (Control cells) or MyD88-silenced TLR4-expressing HEK293 cells. MyD88 appeared at 33kD while β-actin appeared at 42kD. Two independent cytosolic extracts were prepared and run simultaneously for both control and silenced cells. MyD88 band is notably less present (at least 50%) as noted with red arrow in silenced cells.

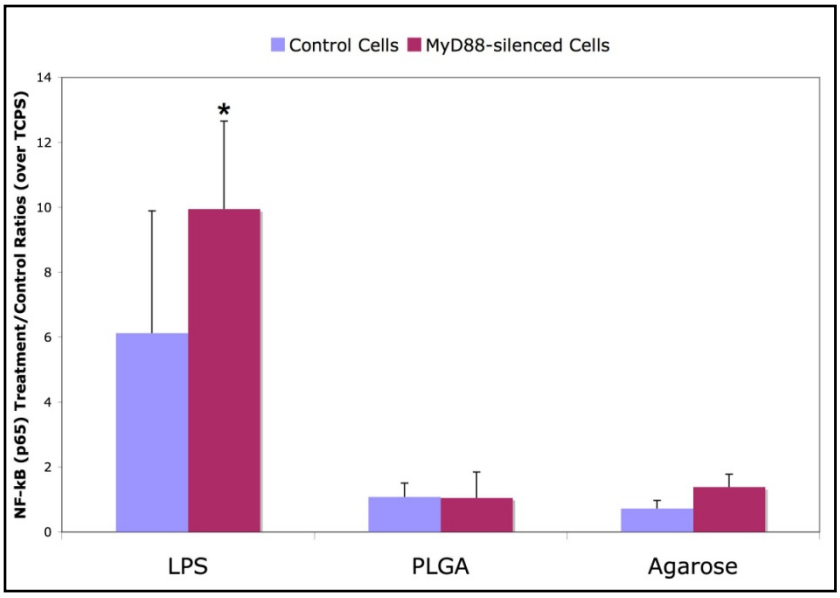

Figure 5-6: Treatment Control ratios of active NF-κB (p65) in TLR4-expressing HEK293 cells or MyD88-silenced TLR4-expressing HEK293 cells. LPS, PLGA or Agarose treated TLR4-expressing HEK293 cells (Control Cells) or MyD88-silenced TLR4-expressing HEK293 cells (MyD88-silenced cells) at 24hr. * indicates $p < 0.05$ in comparison to control (1) (Student t-test), n=3.

To investigate whether the over-expression of TLR4, MD2 and CD14 was necessary for the differential signaling between LPS and biomaterials, wild-type HEK293 cells were cultured and similarly treated with LPS, PLGA films or agarose films for 24hr. Representative family members of AP-1 (cJun) and NF-κB (p65) which were found to be highly activated at 24hr in TLR4-expressing were analyzed following treatment. NF-κB was not activated in response to LPS, PLGA or agarose films in 293 nulls cells (Fig. 5-7A); however, similar to TLR4-expressing 293 cells, wild-type HEK293 cells also had higher levels of AP-1 activation in response to PLGA or agarose films alone (Figure 5-7B).

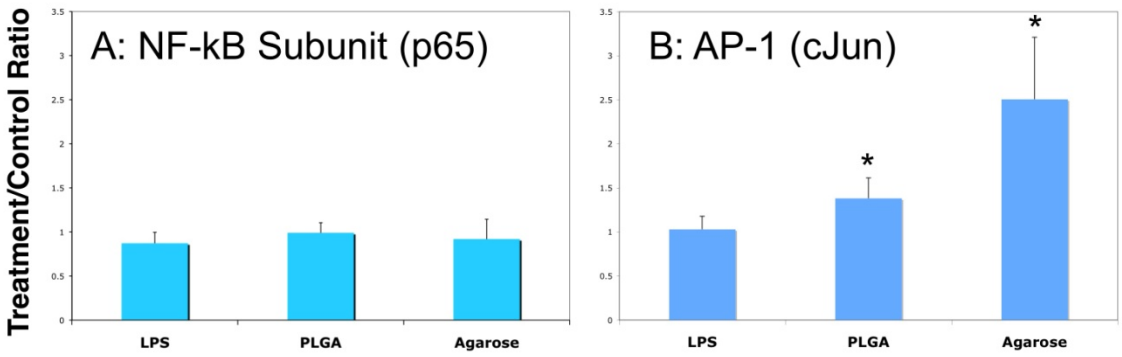

Figure 5-7: Treatment-Control Ratios for active AP-1 in wild-type HEK293 cells. Wild-type HEK293 cells were treated with LPS, PLGA or agarose films for 24hr and active levels of p65 (A) and cJun (B) were determined in nuclear extracts. Treatment control ratios were determined relative to TCPS controls for both subunits. * indicates $p < 0.05$ in comparison to control (1) (Student t-test), n=4.

To examine whether the biomaterial-induced AP-1 activation was yielding increased apoptosis, each cell line (wild-type HEK293 cells TLR4-expressing HEK293 cells and MyD88-silenced TLR4-expressing HEK293 cells) was treated with LPS, PLGA or agarose films or cultured on negative control TCPS for 24hr and assessed for caspase 8 and caspase 3 activation. Across all cell lines examined, agarose treatment induced significant reduction in activated caspase 8 presence (Fig. 5-8). MyD88-silenced TLR4-expressing HEK293 cells was the only cell line to exhibit significant differences in activated caspase 8 presence in cytosolic extract when treated with LPS or PLGA (increases) (Fig. 5-8).

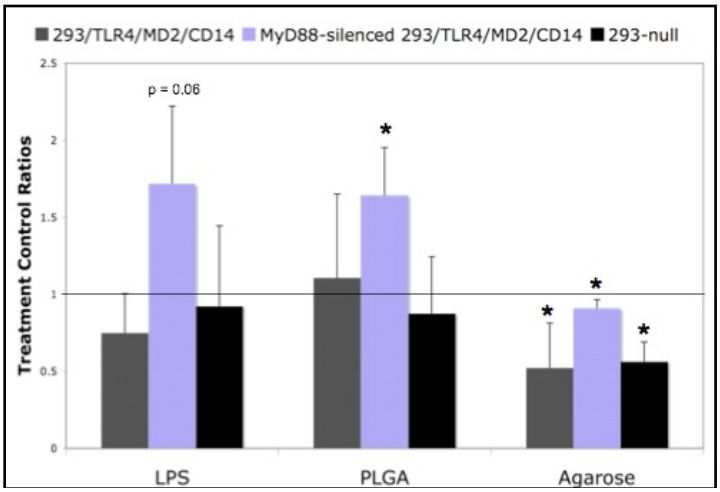

Figure 5-8: Treatment control ratios of activated Caspase 8. LPS, PLGA or Agarose treated TLR4-expressing HEK293 cells, MyD88-silenced TLR4-expressing HEK293 cells or wild-type HEK293 cells (293-null). Star indicates $p < 0.05$ in comparison to control (1) (Student t-test), n=3.

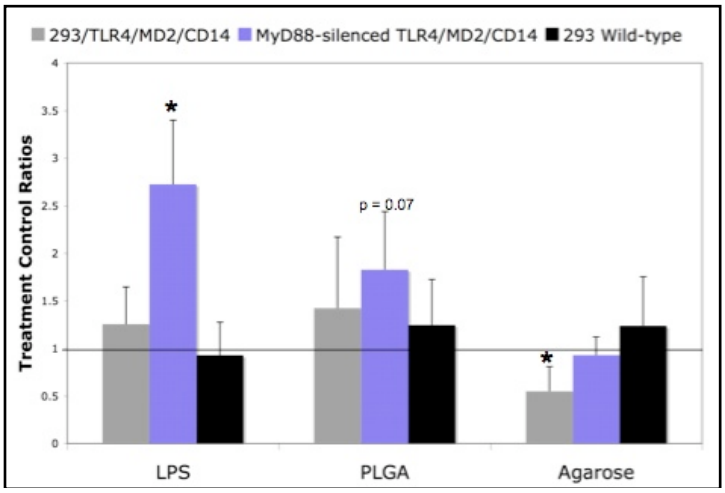

Figure 5-9: Treatment control ratios of activated Caspase 3. LPS, PLGA or Agarose treated TLR4-expressing HEK293 cells, MyD88-silenced TLR4-expressing HEK293 cells or wild-type HEK293 cells. Star indicates $p < 0.05$ in comparison to control (1) (Student t-test), n=3.

Activated caspase 8 cleaves the pro-form of caspase 3 into its activated form, which may then continue the signaling cascade of apoptosis. This was found for the MyD88-silenced cell line which showed that LPS or PLGA treatments induced similar increases in caspase 3 activity (Fig. 5-9) as was found for caspase 8; however, only TLR4-expressing HEK293 cells treated with agarose showed similar reduction in caspase 3 activity as was seen with caspase 8 (Fig. 5-9).

The Role of in TLR4 in the Response of BMDCs to Biomaterials

To isolated the role of TLR4 in biomaterial-induced DC maturation, BMDCs derived from $TLR4^+$ (C57BL/10ScSn) or $TLR4^-$ (C57BL/10ScN) mice were treated with biomaterials PLGA films or MPs for 24hr. Following treatment, non/loosely adherent DCs were harvested and analyzed for expression of CD11c, $I\text{-}A^b$, CD80 and CD86 as well early apoptosis and death via annexin-V and propidium iodide, respectively. In $TLR4^+$ DCs, ultrapure-LPS treatment resulted in increases in expression of CD11c, CD80 and CD86 as compared to iDC (Fig. 5-10). These effects were absent in $TLR4^-$ DCs. $TLR4^+$ DCs treated with PLGA films expressed higher levels of CD86 while similarly treated $TLR4^-$ DCs showed no difference in expression of CD86 as compared to iDC (Fig. 5-10). Both biomaterial treatments also induced signs of early apoptosis as seen in the increases in positive staining with annexin-V, and this response was not found in $TLR4^-$ DCs (Fig. 5-10).

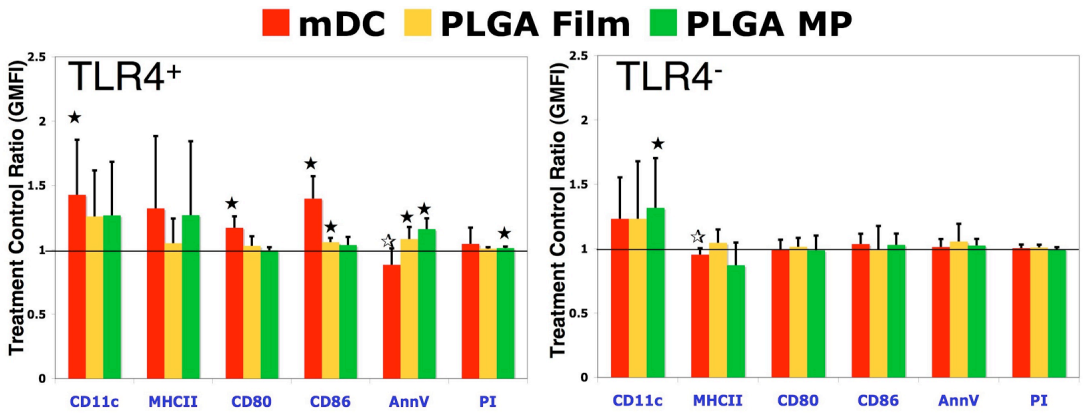

Figure 5-10: Response of TLR4$^+$ (C57BL/10ScSn) or TLR4$^-$ (C57BL/10ScN) BMDCs to ultrapure-LPS (mDC), PLGA film or PLGA MP treatment at 24hr. Geometric mean fluorescence intensities (gMFI) of DCs, as measured by flow cytometry, were determined following treatment. gMFIs of treated DCs were normalized to that of untreated DCs (iDC) and pooled across all trials as Treatment Control Ratios. Data presented as mean+s.d. Statistical analysis was performed by comparing treatment control ratios to 1 (iDC) using Student t-test. ★ indicates greater than iDC, $p < 0.05$; ☆ indicates less than iDC, $p < 0.05$, n=6.

As another measurement of DC maturation induced by ultrapure-LPS, PLGA films or PLGA MP, pro-inflammatory cytokines TNF-α and IL-6 concentrations were measured in cleared supernatants from treated TLR4$^+$ or TLR4$^-$ DCs. TLR4$^+$ DCs, but not TLR4$^-$ DCs, treated with LPS secreted significant amounts of TNF-α (Fig. 5-11) and IL-6 (data not shown). PLGA films or PLGA MPs did not induce any significant production of TNF-α from either TLR4$^+$ or TLR4$^-$ DCs (Fig. 5-11).

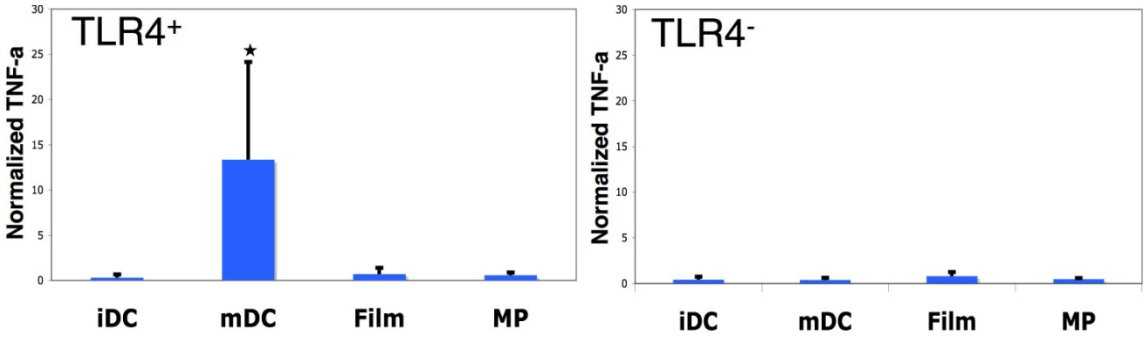

Figure 5-11: Secreted TNF-α concentrations from BMDCs determined from cleared supernatants using ELISA techniques. Concentrations were then normalized to non/loosely adherent DC counts using Coulter Counter. Bars indicate mean+s.d. Normalized TNF-α have units of pg/mL/10^6 cells. ⋆ indicates greater than iDC, ANOVA $p < 0.05$.

BMDCs from another TLR4$^+$ control strain (C57BL/10) or TLR4$^-$ were derived and treated with ultrapure-LPS, PLGA films or MPs for 24hr. Non/loosely adherent BMDCs were collected and assessed for expression levels of I-A^b, CD11c, CD80 and CD86 as well as phosphatidylserine surface presence (annexin-V) and propidium iodide. C57BL/10 DCs treated with ultrapure-LPS (mDC) showed signs of DC maturation as seen by increases in expression of I-A^b, CD80 and CD86 (Fig. 5-12) while biomaterial treatments did not yield this effect. Also, TLR4$^-$ BMDCs, while lacking response to ultrapure-LPS as expected, showed up-regulation of CD80 expression when treated with PLGA films (Fig. 5-12).

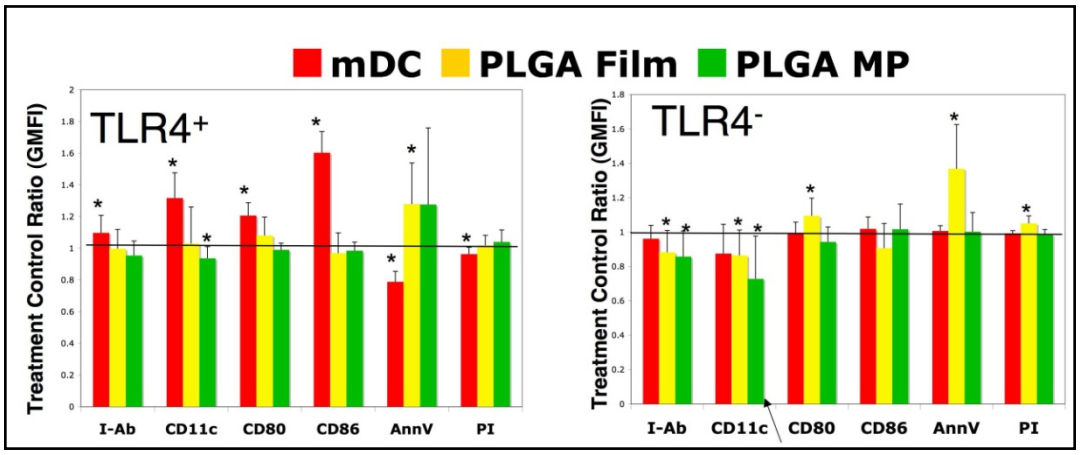

Figure 5-12: Response of BMDCs derived from C57BL/10 or C57BL/10ScN to biomaterials. BMDCs from C57BL/10 (A) or C57BL/10ScN (B) were cultured with ultrapure-LPS (mDC), PLGA films or PLGA MP for 24 hr and assessed for a variety of markers. Results presented are pooled treatment-control ratios over iDC (1) from GMFI values (n=6, except CD11 for TLR4- (arrow) n=5). ★ indicates significantly different from 1, $p \leq 0.05$.

The role of TLR4 in biomaterial-induced maturation was further examined by analyzing both the non/loosely-adherent and adherent BMDC fractions. BMDCs were cultured from C57BL/10 or TLR4^{-} mouse strains and treated as iDC, treated with ultrapure-LPS (mDC) or treated with biomaterials PLGA films or MPs. Unlike adherent DCs from C57BL/6 mice (CHAPTER 6), adherent DCs from the C57BL/10 were not as responsive to PLGA films across all markers, with the exception of an MHC class II molecule (I-A^{b}) (Fig. 5-13). The expression of CD80 and CD86 of adherent DCs from C57BL/10 mice was unaffected by the PLGA film treatment as opposed to similarly analyzed adherent DCs from C57BL/6 mice (Fig. 5-13 vs. Fig. 6-6). The adherent DCs from C57BL/10 mice were more responsive to ultrapure-LPS than the corresponding loosely adherent DCs as seen in CHAPTER 6, and TLR4^{-} adherent DCs did not respond to ultrapure-LPS as expected but yielded mixed results of maturation in response to PLGA films (increased I-A^{b} but decreased CD80) (Fig. 5-13). PLGA MPs induced decreases in I-A^{b} and CD86 expression in adherent BMDCs from both C57BL/10 and TLR4^{-} mice (Fig. 5-13). This is in contrast to adherent DCs from C57BL/6 mice (Fig. 6-6), which showed no significant alteration in DC maturation marker expression in response to PLGA MPs.

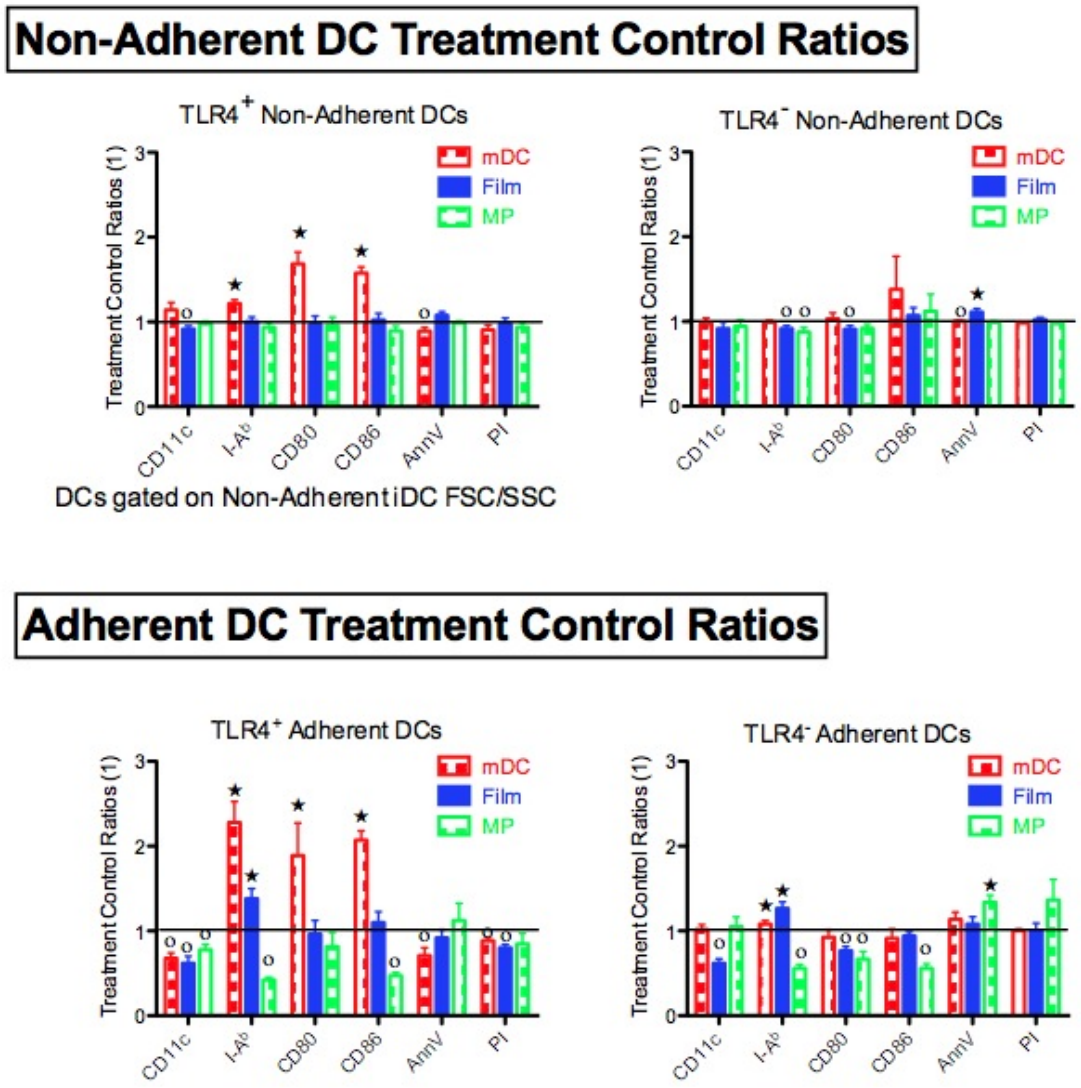

Figure 5-13: Response of Adherent and Non-Adherent BMDCs from C57BL/10 or C57BL/10ScN to biomaterials. $TLR4^+$ (from C57BL/10) or $TLR4^-$ (from C57BL/10ScN) DCs were treated as iDC, mDC (UP-LPS), or PLGA films or PLGA MPs for 24hr. Non-loosely adherent (top) and adherent (bottom) DCs were collected and analyzed via flow cytometer for CD11c, $I\text{-}A^b$, CD80, CD86 expression as well as Annexin V (AnnV) staining for signs of early apoptosis and propidium iodide (PI) staining for necrosis. All n=8 except MP, CD11c for $TLR4^+$ cells (n=7). Star indicates statistically higher than iDC (1), while circle indicates statistically lower than iDC (1), Student t-test, $p \leq 0.05$ NOTE: Data for $TLR4^+$ mice in this Fig. 5-9 the same data used in Fig. 6-6.

TLR-Related Gene Expression in Human DCs in Response to Biomaterials

To examine other TLR/DAMP interactions potentially accounting biomaterial-induced DC maturation, as well as probing TLR-related signaling pathways involved in the response, an RNA expression analysis examining 84 TLR-related genes was investigated. Human monocyte-derived DCs were treated with ultrapure-LPS, PLGA or agarose films for 24hr, and RT-PCR used to examine relative biomaterial-mediated gene expression. Of the 84 genes examined, 33 of them were found to be statistically affected by at least one of the three treatments (Table 5-1). The number of genes affected by ultrapure-LPS treatment was higher than either agarose or PLGA treatment, and numerous TLRs (including TLR4,5,6) were significantly affected by the TLR4-specific ligand (Table 5-1). Following PLGA treatment, across the TLRs, only a significant change in TLR5 expression (down-regulation) was found while agarose was not found to induce any alteration any receptor/surface molecule expression (Table 5-1). Significant differential transcription factor activation was found across the three treatments: LPS induced up-regulation NF-κB family members as well as IRF1, PLGA induced up-regulation of Elk1, and agarose induced up-regulation of cFos (an AP-1 family member).

Significantly affected genes were then used to assess similarity in gene expression patterns between the treatments using hierarchical clustering. It was found that DC treatment with LPS (mDC) or PLGA-induced gene expression patterns that were similar across the three trials investigated as seen in their clustering (Fig. 5-14). Agarose treatment of DCs and iDC gene expression patterns, however, were also found to be similar to each other and showed even tighter clustering (lower nodes/branches) to one other than found between LPS and PLGA treatments (Fig. 5-14).

Table 5-1: Genes which were significantly affected by at least one of the three treatment (ANOVA, $p<0.05$; in comparison to iDC controls), and fold-increase values (treatment over iDC) averaged across the three trials are displayed. Genes were broken into categories and included receptors/surfaces molecules, adaptor/effector molecules, transcription factors and cytokines. Blank table values indicate no significant alteration in gene expression from that of iDC.

Receptors / Surface Molecules

	mDC	PLGA	Agarose
CD80	3.06	-	-
CD86	-	2.17	-
CLEC4E	11.73	-	-
SIGIRR	-12.54	-	-
TLR4	-10.52	-	-
TLR5	-7.5	-5.46	-
TLR6	-4.81	-	-
TLR8	-	-5.9	-
TNFRSF1A	-4.31	-2.59	-

Adaptor/Effector Molecules

BTK	-3.76	-	-
CASP8	-3.25	-	-
IRAK2	3.95	3.1	-
LY86 (MD1)	-5.5	-	-
RIPK2	3.39	-	-
ECSIT	-1.99	-	-1.05
TIRAP	-2.71	-	-
TOLLIP	-2.14	-	1.29
UBE2V1	-4.85	-	-
MAP2K3	-	2.44	-
NFKBIA	-	-2.75	-
PTGS2 (COX2)	-	-	2.74
HRAS	-	3.89	-

Transcription Factors

NFKB1 (p50)	5.88	-	-
REL (cRel)	6.17	-	-
FOS (cFos)	-	-	4.48
ELK1	-	1.49	-
NR2C2	-1.5	-1.81	-
IRF1	4.61	-	-

Cytokines

IL10	-5.26	-	-
IL1A	23.46	-	-
IL1B	44.39	-	-
IL8	45.07	16.79	4.37
TNF-alpha	-1.94	2.36	-

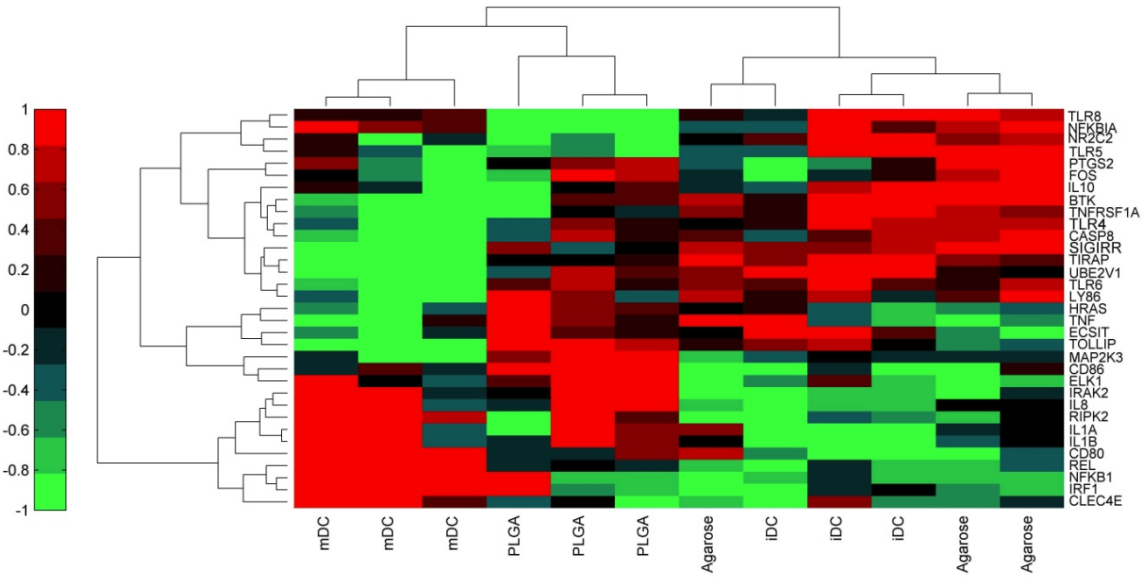

Figure 5-14: Hierarchical 2D cluster analysis of $-\Delta C_t$ for TLR-related gene expression. These were investigated using TLR-SuperArray (SABiosciences) with β-actin and B2M C_t values were used for normalized and $-\Delta C_t$ for each trial and treatment were run through *clustergram* analysis via Matlab.

Purified pooled adherent and non/loosely adherent DCs were then used for whole-cell lysate preparation to determine both total and active transcription factor protein presence. This was done to validate, at the protein level, three transcription factors NFkB1, Elk-1 and cFos responses across each treatment. Total Elk-1 protein was unaffected by LPS or biomaterial treatment at 24hr as similar levels of protein were found across three independent trials in whole-cell DC lysates (Fig. 5-15). This result also matched the level of active Elk1 protein expression found following LPS or biomaterial stimulation which did not alter at 24hr (data not shown).

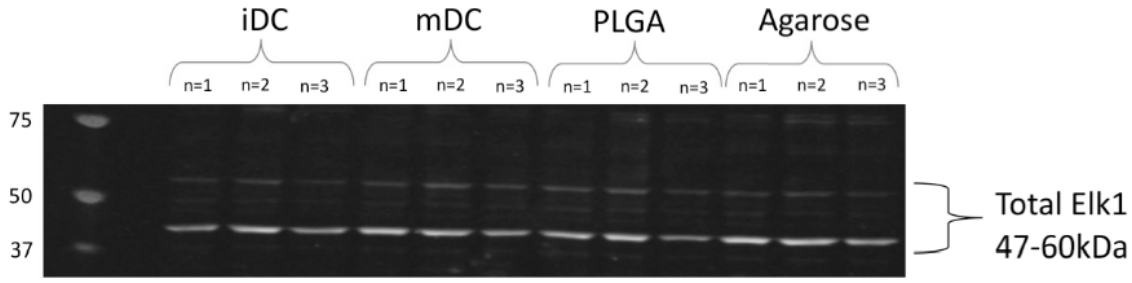

Figure 5-15: Total Elk-1 protein expression from LPS or biomaterial treated whole-cell DC lysates. Elk-1 appears between 47-60kDa. Increased RNA expression of Elk1 found following PLGA treatment did not translate to increase protein levels. Similar results were also found when examining activated Elk-1 (pS383) (data not shown).

Total NFkB1 protein was increased by ultrapure-LPS but not biomaterial treatment at 24hr as seen in higher presence of both active and precursor NFkB1 across three independent trials (Fig. 5-16). This result also matched the level of active NFkB1 protein expression found following ultrapure-LPS or biomaterial stimulation, which also increased for two of the three trials (Fig. 5-17).

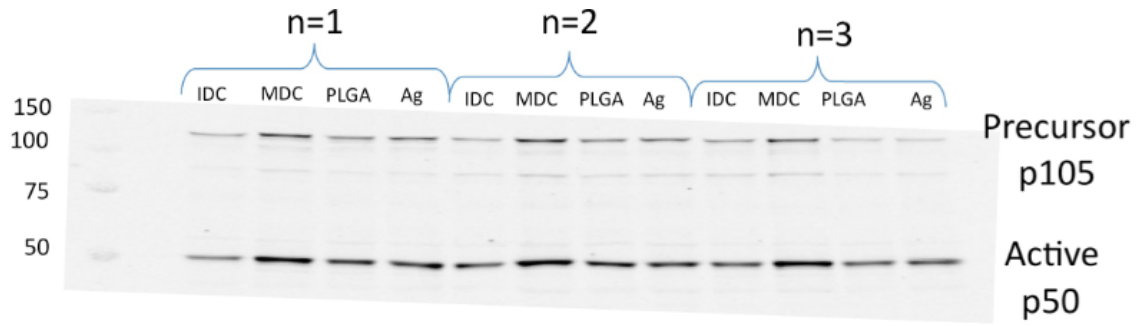

Figure 5-16: Total NFkB1 protein expression from LPS or biomaterial treated whole-cell DC lysates. NFkB1 (both precursor and active version) shows increase in presence follow LPS stimulation (MDC) but not following PLGA or agarose treatment. This concurs with increased RNA expression of NFkB1 found following LPS treatment.

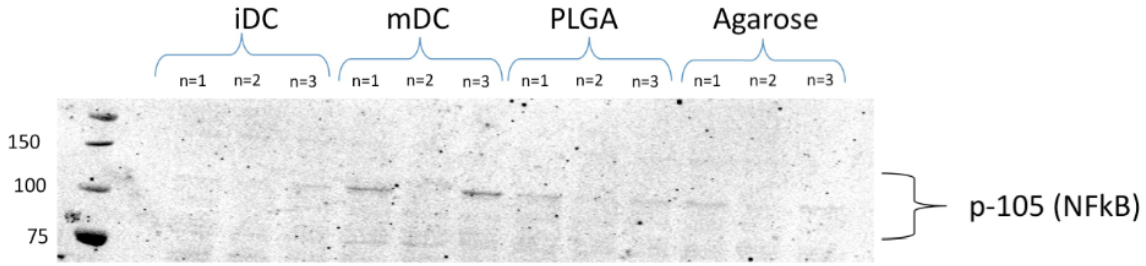

Figure 5-17: Active (phospho-PS939) NFkB1 protein expression from LPS or biomaterial treated whole-cell DC lysates. Activated NFkB1 shows increase in presence follow LPS stimulation (MDC) for two of the three trials but not following PLGA or agarose treatment. This concurs with increased RNA expression of NFkB1 found following LPS treatment.

Total cFos protein was unaffected by LPS or biomaterial treatment at 24hr as similar levels of protein were found across three independent trials (Fig. 5-18). This result also matched the level of active cFos protein expression found following LPS or biomaterial stimulation which did not alter at 24hr (data not shown).

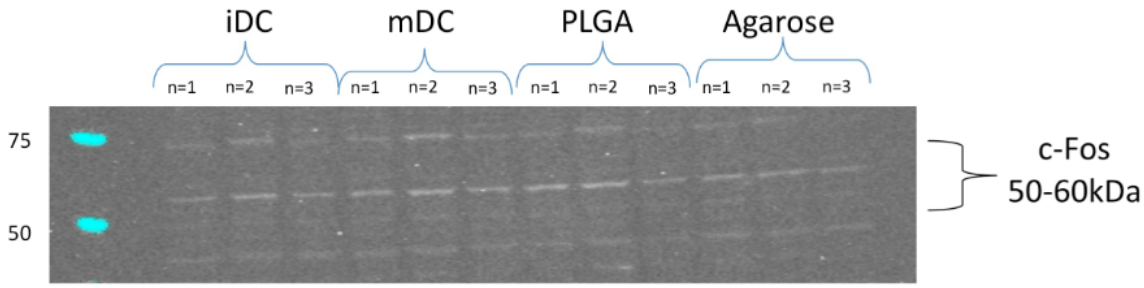

Figure 5-18: Total cFos protein expression from LPS or biomaterial treated whole-cell DC lysates. cFos protein presence shows no significant alteration follow LPS or biomaterial stimulation for 24hr. Agarose-induced increases in cFos RNA expression were not translated at 24hr to increases in cFos protein expression. Similar results were found for activated (pT235) cFos (data not shown).

Discussion

In order to isolate the role of TLR4-induced signaling in the potential cellular recognition of biomaterial-presented 'danger signals', TLR4-expressing HEK293 cells were treated with PLGA or agarose films as well as LPS, and the levels of activated NF-κB and AP-1 was assessed. The high levels of activated NF-κB and AP-1 found in nuclear extracts from TLR4-expressing HEK293 cells in response to LPS was expected (Fig. 5-1, Fig. 5-3), as was their induced secretion of IL-8 secretion (Fig. 5-2). Biomaterial treatments, however, seemed to preferentially stimulate AP-1 signaling pathway only as both PLGA and agarose treatments resulted in significantly higher levels of activated AP-1 family members (and not NF-κB family members) at 24hr (Fig. 5-1, Fig. 5-3). To assess the TLR4 specificity in TLR4-expressing HEK293 cells, a stably silenced transform was created to knockdown MyD88, an important adaptor molecule in TLR signaling (Fig. 5-4 and Fig. 5-5) and was intended to inhibit TLR4-induced signaling. However, MyD88-silenced TLR4-expressing HEK293 cells were still responsive to LPS as seen in significantly increased NF-κB activation, which was similar to levels found in non-silenced TLR4-expressing HEK293 cells (Fig. 5-6). Therefore, it was not possible to prevent TLR4-induced signaling by silencing MyD88. This may be due to either incomplete knockdown, as noted in Fig. 5-5, or due to the involvement of another adaptor molecule Toll/IL-1R domain-containing adapter molecule (Trif, illustrated in Fig. 3-2) which can cross-talk with the NF-κB pathway (Takeda et al. 2003; Akira and Takeda 2004; Kawai and Akira 2006) and allow for TLR signaling propagation to by-pass MyD88.

Due to wild-type HEK293 cells expressing little to no TLR4 protein (Devaney et al. 2003), they were used as a TLR4-deficient control to examine their response to LPS, PLGA or agarose treatment. As expected, wild-type HEK293 cells did not respond to

LPS as seen in lack of both activated NF-κB and AP-1 in nuclear extracts at 24hr (Fig. 5-7) verifying the TLR4 specific response. However, while wild-type HEK293 cells showed no significant NF-κB activation (p65) across all treatments (Fig. 5-7A), they showed significant increase in the level of activated cFos (AP-1) present in their nucleus at 24hr following PLGA or agarose treatment (Fig. 5-7B). Thus, the over-expression of TLR4 in HEK293 cells was not necessary for the biomaterial-induced AP-1 response (Fig. 5-3).

NF-κB and AP-1 signaling pathways regulate one another and following cell stimulation both NF-κB and AP-1 activation may be necessary for cell survival (Kriehuber et al. 2005). Unbalanced AP-1 activation, as occurred in biomaterial-treated HEK293 cells, may have resulted in increased cellular apoptosis. Signs of biomaterial-induced apoptosis in TLR4-expressing HEK293 cells, MyD88-silenced TLR4-expressing HEK293 cells and 293 null cells were investigated by examining the level of both activated caspase 8 (early apoptosis marker) and activated caspase 3 (late apoptosis marker) in each cell line. Unexpectedly, agarose, which induced the highest level of AP-1 activation, induced significantly lower levels of activated caspase 8 present across all cell lines at 24hr indicating lower levels of apoptosis (Fig. 5-8). Since caspase 8 cleaves the pro-form of caspase 3 into its activated form, one would expect the trends seen with activated caspase 8 in biomaterial-treated cells to also be seen when examining activated caspase 3. However, only agarose-induced lowering of activated caspase 8 presence propagated to caspase 3 in TLR4-expressing HEK293 cells while no significant alteration in activated caspase 3 presence was found in agarose treated MyD88-silenced TLR4-expressing HEK293 cells or wild-type HEK293 cells (Fig. 5-9). Therefore, it appeared that AP-1 activation did not correlate directly to an increase in apoptosis for both markers examined. Caspase activation, however, may not be the most appropriate marker for biomaterial-induced apoptosis in HEK293 cells as other

apoptotic markers such as DNA fragmentation detection via terminal transferase dUTP nick end labeling (TUNEL) may be necessary.

$TLR4^-$ mice were used as a source of BMDCs to isolate the role of TLR4 in biomaterial-induced DC maturation. BMDCs from $TLR4^+$ or $TLR4^-$ mice were treated with ultrapure-LPS (a TLR4-specific ligand) or treated with PLGA films or MPs which have previously been shown to induce signs of BMDC maturation (Yoshida et al. 2007). The isolation of TLR4-induced activation using $TLR4^+$ and $TLR4^-$ was verified by the treatment of ultrapure-LPS, which only induced signs of activation (increased MHC class II, CD80 ad CD86) in the non/loosely adherent BMDC population from $TLR4^+$ but not $TLR4^-$ mice (Fig. 5-10). Furthermore, $TLR4^+$ non/loosely adherent BMDCs were able to respond and mature in the presence of PLGA films which induced signs of DC maturation (increased CD86 expression), and this is absent in $TLR4^-$ BMDCs possibly implicating a role for TLR4 in biomaterial-induced DC maturation (Fig. 5-10). Treatment with PLGA MPs, however, did not induce signs of DC maturation in non/loosely adherent BMDCs from either mice strain (Fig. 5-10). Also, while ultrapure-LPS treatment induced production of TNF-α in a TLR4-dependent manner, neither PLGA film nor MP treatment resulted in the significant secretion of the pro-inflammatory cytokine from $TLR4^+$ or $TLR4^-$ BMDCs (Fig. 5-11) which had previously been seen with similarly treated BMDC from C57BL/6 mice (Yoshida et al. 2007). Both biomaterial treatments, however, induced signs of early apoptosis as seen in the increased positive staining of annexin-V, and this response was not found in $TLR4^-$ BMDCs, which indicated a TLR4-dependent response (Fig. 5-10). Interestingly, while PLGA films induced increased expression of CD86 in $TLR4^+$ BMDCs they simultaneously induced increased signs of apoptosis, both in a TLR4-dependent manner. This is in contrast to ultrapure-LPS treatment, which resulted in increased DC maturation and decreased signs of early apoptosis (Fig. 5-10). A similar response has also been found with $NiCl_2$, which induced DC maturation and subsequent

DC allogeneic T-cell stimulation with a simultaneous induction of DC apoptosis (Manome et al. 1999). This response was speculated to be dependent on ERK, as ERK-inhibited DCs have impaired LPS-induced annexin-V staining without affecting DC maturation (Rescigno et al. 1998). Thus, increased activation through ERK pathway by PLGA treatment, may offer an explanation to the differential BMDC response between biomaterial and LPS treatments.

The response of BMDCs from $TLR4^{+}$ mice (again, the C57BL/10ScSn strain) to the ultrapure-LPS (as well as PLGA) was not as robust as had previously been seen with BMDCs from C57BL/6 mice (Yoshida et al. 2007). Therefore, this study was repeated using another appropriate TLR4-expressing strain, C57BL/10, as a source of BMDCs to examine if this strain would be a more sensitive control strain for $TLR4^{-}$ mice. Non/loosely adherent BMDCs from C57BL/10 did not appear to be responsive to PLGA film or MP treatment while they were still responsive to the ultrapure-LPS stimulation as seen with TLR4-depedent DC maturation marker increased expression for BMDCs from C57BL/10 mice (Fig. 5-12). However, non/loosely adherent iDCs collected appeared to be pre-activated as assessed by $I\text{-}A^{b}$ expression (described in detail, CHAPTER 6). Therefore, characterization of both adherent and non/loosely adherent cell in BMDC culture was assessed, which revealed that adherent BMDCs were more responsive to stimuli than non/loosely adherent BMDC due to maintaining an immature status (CHAPTER 6).

The role of TLR4 in biomaterial-induced DCs was thus further investigated with analysis of both adherent and non/loosely adherent treated BMDC fractions from C57BL/10 or $TLR4^{-}$ mice. Overall the effects of all treatments on non/loosely adherent DCs were less dramatic than seen in adherent BMDCs. However, the effects of the maturation-inducing stimulus, PLGA film induced increase in CD80/86 expression), on adherent BMDCs from C57BL/6 (Fig. 6-6) was not found with BMDCs from C57BL/10

strain (Fig. 5-13 and Fig. 6-6). The TLR4^{-} adherent BMDCs actually showed signs of maturation in response to PLGA films as seen with a significant increase in I-A^{b} which was not found with their non/loosely adherent counterpart (Fig. 5-13). However, TLR4^{-} adherent BMDCs also responded to PLGA films by significantly down-regulating CD80 expression (Fig. 5-13). Therefore, there was contrasting evidence as to whether biomaterial-induced DC maturation was TLR4-dependent as TLR4^{-} BMDCs showed signs of both maturation and becoming more immature. It may be possible that TLR4 knockout (KO) strains developed by Shizuo Akira (Hoshino et al. 1999) and not TLR4-hyporesponsive strains or TLR4-deficient (C3H/HeJ or C57BL/10ScN, respectively) (Poltorak et al. 1998) are more suitable for determining the role of TLR4 in biomaterial-induced DC maturation due to the use of C57BL/6 as a background strain. The difference in background strain, of which genetic differences have been found between C57BL/6 and C57BL/10 (McClive et al. 1994), may allow for more prominent differences between biomaterial-mediated responses of BMDCs from the TLR4^{+} and the TLR4-KO strain. This is supported by a recent study which was successful in showing a role for TLR4 and TLR2 in the response of BMDCs to PLGA using TLR4$^{-/-}$ mice of C57BL/6 background (Shokouhi et al. 2010). This may allow for increased sensitivity for BMDC response to biomaterials. However, another possible explanation for the less than anticipated response to biomaterials from all TLR4^{+} mice investigated may be due to analysis of a 24hr time point following stimulation. Earlier time points (such as 5 or 12hr) may potentially allow for increased sensitivity for determining biomaterial-induced BMDC maturation.

TLR-related gene expression was examined to determine the effects of both LPS and biomaterial treatment on both specific TLR down-regulation, which may indicate a role in recognition, and overall gene expression patterns, which would determine if biomaterial treatment induced similar gene regulation to that of PAMP stimulation.

Ultrapure-LPS influenced a larger number of individual genes with altered expression than PLGA, which in turn also significantly affected a larger number of genes investigated than agarose (Table 5-1). PLGA is known to induce DC maturation to a similar degree to that of LPS (Yoshida and Babensee 2004; Babensee and Paranjpe 2005; Yoshida and Babensee 2006) and here, the overall gene expression pattern induced by PLGA matched that of LPS more than either iDC or agarose treatment (Fig. 5-14). Also, iDC and agarose treatments clustered tightly together which is expected as agarose does not tend to induce DC maturation (Babensee and Paranjpe 2005; Yoshida and Babensee 2006). These results further verify the activating (PLGA) or non-activating (agarose) potential for biomaterial control of an immune response by directing DC maturation through global gene expression manipulation. Furthermore, ultrapure-LPS treatment significantly affected expression of several TLRs (TLR4, TLR5 and TLR6) indicating that the TLR4-specific stimulation resulted in other non-specific TLR down-regulation (Table 5-1). Therefore, conclusions cannot be drawn on the role of specific TLRs to recognize biomaterials through gene expression analysis alone. So while PLGA treatment induced significant decreases in TLR5 and TLR8 expression (Table 5-1) and not TLR4 or TLR2 as was anticipated due to their known role in the recognition of 'danger signals', the role of TLR5 and TLR8 (Table 5-1) in the response of DCs to PLGA is inconclusive potentially due to compensatory down regulation of multiple TLRs. Also, gene expression at the mRNA level does not always translate to protein expression, as noted below for the transcription factor protein validation. This implies that though TLRs may be significantly down-regulated in the transciptome, TLR protein expression and subsequent role in recognition of biomaterial may not be correlated to expression at the mRNA level.

Interestingly, differential transcription factor expression appeared across the three treatments. Ultrapure-LPS induced increases in NFkB1, PLGA induced increases

in Elk1 and agarose significantly increased cFos (AP-1) expression. LPS-induced NF-κB expression was anticipated as LPS increased NF-κB activation in TLR4-expressing HEK293 cells (Fig. 5-1). Also, an increase in cFos expression in DCs following agarose treatment matches similarly treated HEK293 cells (Fig. 5-7) implying that agarose potentially affects AP-1 pathway across many cell types. As previously mentioned, BMDC apoptosis caused by PLGA (Fig. 5-10) may potentially be linked to ERK activity (Rescigno et al. 1998). PLGA induced a significant increase in Elk-1 expression in human DCs (Table 5-1), which is directly controlled and activated by ERK (Cruzalegui et al. 1999). Therefore, it is possible that PLGA stimulated ERK/Elk-1 pathway and may explain its effect on DC apoptosis.

Gene (RNA) expression confirmation at the protein level was determined by examining both total and activated NFkB1, cFos and Elk-1 presence. NFkB1 RNA expression was increased at 24hr following LPS treatment only, and protein levels (both total and active forms of NFkB1) were found to match these findings (Fig. 5-16 and Fig. 5-17). However, increased Elk-1 RNA expression in human DCs following PLGA treatment and increased cFos RNA expression following agarose treatment were not validated at the protein level examining either total or active forms of the two transcription factors (Fig. 5-15 and Fig. 5-18). Therefore, these biomaterial-induced RNA responses were not translated to protein at 24hr. It is possible that only NFkB1 protein expression showed an increase at 24hr due to persistent activation while cFos and Elk-1 might show increases in protein expression at an earlier time point than assessed in this study. However, all HEK293 cell line examined showed increased levels of active AP-1 (cFos) in nuclear extracts following agarose stimulation at 5h and even more significantly at 24hr. Therefore, it may be that nuclear extraction from DCs may be necessary for analysis of specific transcription factor activation levels to note the differences across biomaterial treatments as opposed to whole-cell extract which was analyzed herein.

In conclusion, we sought to determine the role of TLR4/ 'danger signal' interactions in the response of DCs to biomaterials. Biomaterial treated HEK293 cells resulted in increases in AP-1 activation, which was found to be independent of TLR4 expression. A role for TLR4 in biomaterial-induced DC maturation was shown in BMDCs treated with PLGA films, which showed TLR4-dependent signs of maturation and apoptosis. Further analysis of adherent BMDCs treated with PLGA revealed inconclusive evidence as $TLR4^{-}$ BMDCs showed signs of both increased and decreased DC maturation. Lastly, global gene expression analysis of PLGA and LPS treated human DCs revealed similarity indicating a comparable response to both the activating biomaterial and PAMP. However, key differences in this response may be dictated by their induction of differential transcription factors.

CHAPTER 6

ADHERENT FRACTION OF BONE MARROW-DERIVED DENDRITIC CELLS IS MORE RESPONSIVE TO LPS AND BIOMATERIAL STIMULI THAN THE NON/LOOSELY ADHERENT DENDRITIC CELL FRACTION [*]

Introduction

Dendritic cells (DC) act as the sentinel cells of the body by constantly surveying their environment for foreign entities. Upon such an encounter, immature DCs (iDCs) may respond innately to pathogens through pattern recognition receptors (PRR), which induce their differentiation into mature DCs (mDC). An mDC may then migrate to a lymph node which allows for initiation of an adaptive immune response through co-stimulation and MHC class II presentation of the pathogen by-products to T cells (Banchereau and Steinman 1998). Immature DCs may also play a role in the immune response through induction of self-tolerance by presenting self-peptides to T cells in the absence of additional stimuli (Hawiger et al. 2001; Steinman and Banchereau 2007). Thus, DCs may control both the occurrence and absence of an immune response.

For these reasons, DCs have been investigated for use in therapies ranging from cancer vaccines to amelioration of autoimmune diseases (Ludewig et al. 2001; Ueno et al. 2010). In laboratory work investigating these therapies, murine conventional DC (cDC) cultures are used. Typically, murine cDCs are derived from BM progenitor cells (Inaba et al. 1992; Lutz et al. 1999) but have also been derived from blood mononuclear cells (Agger et al. 2000) through differentiation using GM-CSF alone or in combination with IL-4. Following cultures, which may vary in time frame, non/loosely adherent DCs are routinely collected as iDCs. In the case of DCs differentiated with both GM-CSF and IL-4, DCs have been shown to express higher levels of CD11c (Masurier et al. 1999), a

[*] Modified from Rogers T, Babensee JE "Characterization Of Adherent And Non/Loosely Adherent Murine Bone Marrow Derived Dendritic Cells And Their Responsiveness To Maturation Stimuli" (Manuscript submitted J Immunol Methods)

mouse cDC marker, as well as possessing higher potency during mixed lymphocyte reaction (Mayordomo et al. 1997), than with GM-CSF alone. They can then further be purified to greater than 90% using CD11c magnetic-bead isolation (Menges et al. 2002; Yoshida et al. 2007).

Previously, we have shown that certain biomaterials (e.g. PLGA, chitosan) may act as an adjuvant to enhance, while others may minimize (e.g. agarose), the immune response to a co-delivered biologic (Matzelle and Babensee 2004; Norton et al. 2010), to an extent which mimics the respective level of induced DC maturation (Yoshida and Babensee 2006; Yoshida et al. 2007). For BMDCs, the collection, treatment and analysis solely of the non/loosely adherent cell population is common. Many investigators often overlook the underlying adherent BMDC population. Adherent BMDCs are typically only examined when studying the effects of substrates which may directly influence adhesion such as extracellular matrix proteins (Acharya et al. 2008). However, on tissue culture polystyrene (TCPS) alone, adherent rat BMDCs were found to express high levels of IL-10 and CD54 and seemed capable of inducing tolerance upon reinjection in contrast to their non/loosely adherent BMDC counterpart which maintained immunity (Yang et al. 2000). Adherent rat BMDCs had a higher phagocytic capacity and lower MHC class II and CD86 surface presence than the non/loosely adherent BMDC counterpart (Muthana et al. 2004) while adherent mouse BMDC injections (and not non/loosely adherent BMDCs) have allowed prolongation of heart allografts (Peche et al. 2005). Thus, there is evidence that the phenotype of adherent BMDCs is more immature (or even tolerogenic) than the non/loosely adherent BMDC population.

Presented here is a characterization of the cellular phenotypic profiles across the culture time frame of both adherent and non/loosely adherent DCs derived from different strains of murine BM. Specific cellular identification markers were examined as well as analysis of the maturation state of both adherent and non/loosely adherent BMDCs at

the initial time point of BM collection through the endpoint time of the culture following a 24h treatment with PLGA films, PLGA MPs or LPS. The results indicate that not only are adherent BMDCs more “immature,” but they are in fact more receptive to stimuli than the non/loosely adherent BMDC population.

Materials and Methods

Mice Strains

Male mice (6 weeks in age) of two strains were used as the source of BM for the characterization study: C57BL/10 (n=3) and C57BL/10ScSn (n=3) (The Jackson Laboratory, Bar Harbor, ME). Both strains are relevant control strains for the toll-like receptor 4 (TLR4)-deficient mice C57BL/10ScN (Poltorak et al. 1998). For biomaterial treatments, C57BL/6 (n=7-8) and C57BL/10 (n=7-8) mice were used (The Jackson Laboratory).

Bone Marrow Derived Dendritic Cell Culture

The BMDC culture method used here is based on a previously established method with modifications (Inaba et al. 1992; Yoshida et al. 2007). Femur and tibia collection (as well as animal housing) was performed at Emory University Whitehead animal facility (IACUC protocol 044-2006). For culture protocol, please see method section of CHAPTER 5 (BMDC culture), and Fig. 6-1. Immature DCs collected on Day 6 were induced into mDC through treatment with ultrapure-LPS at 1μg/mL or left untreated as a negative control for iDC.

Characterization Procedure

At specific time points of BMDC culture (see Fig. 6-1), both adherent and non/loosely adherent fractions were collected and analyzed separately. Non/loosely adherent DCs were collected by swirling plates 10 times clockwise, 10 times counter clockwise, 10 times left/right and 10 times up/down followed by gentle pipette collection and counting using Coulter Counter (Multisizer 3, Beckman Coulter, Brea, CA). Immediately following, cell dissociation solution (Sigma) was added to wells (3mL for 6 well plates, and 1mL for 24 well plate) and placed in 37^{o}C incubator until remaining

adherent DC dissociation occurred following gentle tapping and pipetting. Adherent DCs were then collected and counted using Coulter Counter. Both adherent and non/loosely adherent DCs were rinsed with FACS buffer (96% (v/v) Hank's HEPES Buffer (120mM NaCl, 10mM KCl, 10mM $MgCl_2$, 10mM glucose, and 30mM HEPES. All chemicals from Sigma), 1% (v/v) human serum albumin (HSA) (EMD Biosciences, La Jolla, CA), and 1.5mM $CaCl_2$). Both collected populations were then double stained for 1hr in FACS buffer using the following pairs of antibodies, which recognize the noted murine molecules (Table 6-1). Flow cytometry was performed using LSR II (BD Biosciences). Geometric mean fluorescence intensity (gMFI) was used determined using FloJo software (Tree Star, Inc, Ashland OR) to quantify expression levels on gated (FSC/SSC) DC populations as necessary.

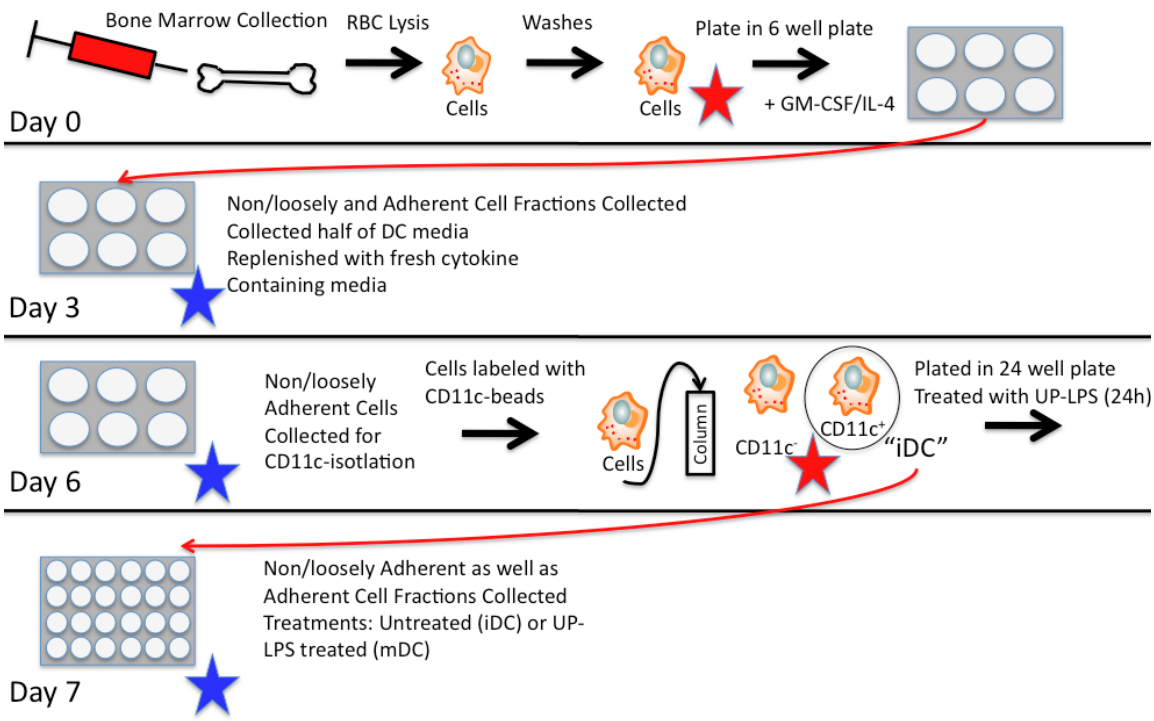

Figure 6-1: BMDC culture and characterization time points. Red star indicates characterization procedure performed by flow cytometry on cell suspensions. Blue star indicates flow cytometry analysis of both non/loosely adherent and adherent cells which were collected and also counted to measure percentage of adherent cells. Both fractions were stained with each of the following pairs of antibodies listed in Table 6-1. On Day 0, cell suspension prior to plating was analyzed. On Day 6, pre-sorted cells (adherent and non-adherent) and $CD11c^+$ isolation fractions were characterized. On Day 7, cells that were left untreated (iDC) or treated with ultrapure-LPS (mDC) were also characterized.

Table 6-1: Antibodies and markers used for flow cytometry characterization. Throughout BMDC culture time frame, presence of cell types were probed for using cell specific markers as noted in table. Cells were double stained with APC/PE antibodies as denoted by markers that are paired together (e.g. CD80-APC, CD86-PE). Clones, isotypes and vendor information is also provided.

Cell Type	MARKER	CLONE	Isotype	Vendor
B Cell	CD19-PE	6D5	Rat IgG2a	Miltenyi Biotec
T Cell	CD3-APC	145-2C11	Ham IgG1	Miltenyi Biotec
RBC	TER119-APC	Ly-76	Rat IgG2b	Miltenyi Biotec
Macrophage	F4/80-PE	BM8	Rat IgG2a	eBioscience
Monocyte	CD14-APC	Sa2-8	Rat IgG2a	eBioscience
Platelets	CD41-PE	MWReg20	Rat IgG1	eBioscience
DC	CD11c-APC	HL3	Ham IgG1	BD Bioscience
DC/B Cell	MHCII-PE	AF6-120.1	Ms. IgG2a, k	BD Bioscience
DC/B Cell	CD80-APC	16-10A1	Ham IgG2	BioLegend
DC/B Cell	CD86-PE	GL1	Rat IgG2a	BioLegend
Plasmacytoid-DC	PDCA-1-APC	JF05-1C2.4.1	Rat IgG2b	Miltenyi Biotec
NK Cells	CD49b-PE	DX5	Rat IgM	Miltenyi Biotec
Granulocytes	GR-1-APC	RB6-8C5	Rat IgG2b	Miltenyi Biotec
Stem Cell	CD117-PE	3C1	Rat IgG2b	Miltenyi Biotec
Fb/Epithel	CD121a-PE	35F5	Rat IgG1	BD Bioscience
Plasma Cells	CD138-APC	281-2	Rat IgG2a	BD Bioscience

Biomaterial Preparation and Treatment

A solution of 10% w/v PLGA (mole ratio 75:25, inherent viscosity 0.70 dL/g in trichloromethane; Durect/Lactel Absorbable Polymers, Birmingham, AL) was prepared in 20mL dichloromethane overnight in sterile polypropylene tubes (Yoshida and Babensee 2004). To form films, this solution was gently poured into a cleaned 100mm Teflon dish and allowed to sit for two days in fume hood until DCM had evaporated. PLGA films of appropriate sizes were prepared using a flame-sterilized punch. Punched films were washed for one hour in endotoxin free water (Lonza, Basel, Switzerland).

PLGA MPs were prepared as described in CHAPTER 5 using a single emulsion solvent evaporation technique (Yoshida and Babensee 2004).

Endotoxin content of biomaterials was examined using the Limulus Amebocyte Lysate assay (QCL1000, Lonza) with the chromogenic substrate added in the presence of the materials. Endotoxin levels of all biomaterials used tested below 0.2 EU/mL.

CD11c-isolated DCs (on day 6 of culture) were prepared from C57BL/10 or C57BL/6 mice and left untreated as iDC, or treated with ultrapure-LPS for mDCs, or treated with PLGA films or MPs for 24h. Following treatment, non/loosely adherent BMDCs as well as adherent BMDCs were collected. Cells were analyzed with flow cytometry after being double stained for I-A^b/CD11c or CD80/CD86 and gMFIs determined. Treatment control ratios were determined by dividing gMFI received following LPS or biomaterial treatment by the gMFI for iDC. Normalization was separate for the adherent and non/loosely adherent iDC populations.

Statistical Analysis

For most statistical analysis, an ANOVA analysis was performed (GraphPad, LaJolla, CA) using Tukey-post test ($p<0.05$) with mice nested within treatment. When

comparing data to a normalized value relative to iDCs (hence comparing to 1), a two-sided Student t-test was performed ($p \leq 0.05$)

Results

Day 0 Cellular Characterization

The cell populations present during development of cDCs from C57BL/10 or C57BL/10ScSn mouse BM were characterized as well as the level of pre-activation in both the adherent and non/loosely adherent BMDC fractions. On Day 0, following removal of RBCs from BM, the majority of cells from both strains stained positive for a granulocyte marker (Gr-1) or B cell marker (CD19) (Table 6-2). Also, BM cells stained positive to varying degrees for a co-stimulatory molecule (CD80), a macrophage marker which is also present during myeloid development (F4/80) (Leenen et al. 1994), an MHC class II molecule (I-A^b), and a platelet/megakaryocyte marker (CD41) (Table 6-2). There was no detection of expression of CD3 (T cells), TER119 (RBCs), CD14 (monocytes), PDCA-1 (plasmacytoid DCs), CD49b (natural killer cells), CD117 (stem cells), CD121a (various cell types including fibroblasts) or CD138 (plasma cells) on cells from Day 0 through the end of BMDC culture for both strains.

Table 6-2: Flow cytometry analysis for surface markers during BMDC culture. For Day 0, range of the percentage of total gated BM cells that were positive for stated marker are given (n=3 for both C57BL/10 and C57BL10ScSn strains). Percentages given were determined by the low and high values for pooled data for both strains. For Day 3, 6 and 7 relative expression levels for markers on stated for both the adherent and non-adherent fraction of DCs. On Day 6, levels post-CD11c isolation are also given.

Day 0		Day 3				Day 6					Day 7				
	Bone Marrow		**Non-Adherent**	**Adherent**			**Non-Adherent**	**Adherent**	**Post-Isolation**			**Non-Adh (iDC)**	**Non-Adh (mDC)**	**Adherent (iDC)**	**Adherent (mDC)**
Gr-1$^+$	30-40%	**F4/80**	Mid	Mid		**F4/80**	Mid	Mid	Mid		**F4/80**	Mid	Mid	Mid	Mid
CD19$^+$	20-25%	**CD41**	Low	Low		**CD41**	Low	Mid	Low		**CD41**	Low	Low	Mid	Mid
CD80$^+$	10-15%	**CD11c**	Low	Mid		**CD11c**	Mid	High	Mid		**CD11c**	Low	Low	Mid+Low	Mid+Low
F4/80$^+$	20-40%	**I-A^b**	High	High+Low		**I-A^b**	High+Low	Low	High-Low		**I-A^b**	High	High	Low	High
I-Ab$^+$	23-31%	**CD80**	High	Low		**CD80**	Mid	Low	Mid		**CD80**	High	High	Low	Mid
CD41$^+$	20-27%	**CD86**	High+Low	Low		**CD86**	High+Low	Low	High+Low		**CD86**	High	High	Mid	High

Adherent and Non/loosely Adherent BMDC Characterization Prior to Treatment

By Day 3, cDCs ($CD11c^+$) appeared in the culture in both adherent and non/loosely adherent fractions (Table 6-2, Fig. 6-2A for C57BL/10 and Fig. 6-3A for C57BL/10ScSn). There were few cells that stained positive for granulocyte or B cell markers that had been present on Day 0 (not shown). BMDCs on Day 3 began to show signs of differential expression levels of co-stimulatory molecules CD80 and CD86 between both the adherent and non/loosely adherent DC populations. While non/loosely adherent DCs expressed high levels of an MHC class II molecule ($I\text{-}A^b$), CD80 and CD86, adherent DCs expressed lower levels of these markers (Table 6-2, Fig. 6-2B for C57BL/10, and Fig. 6-3B for C57BL/10ScSn). Adherent DCs, however, did express higher levels of CD11c than non/loosely adherent DCs (Table 6-2, Fig. 6-2A for C57BL/10 and Fig. 6-3A for C57BL/10ScSn).

By Day 6, expression of granulocyte or B cell markers did not appear to be present in either adherent or non/loosely adherent cell fractions (data not shown). The levels of maturation markers between the two BMDC fractions differed in a similar manner as had been observed on Day 3 in that the non/loosely adherent BMDC displayed high levels of CD80, CD86 and MHC class II ($I\text{-}A^b$) expression while adherent BMDCs exhibited low levels of expression of these markers (pre-isolation, Table 6-2, Fig. 6-2A,B for C57BL/10 and Fig. 6-3A,B for C57BL/10ScSn). Adherent BMDCs maintained higher levels of CD11c expression than non/loosely adherent BMDCs (Fig. 6-2A for C57BL/10 and Fig. 6-3A for C57BL/10ScSn). The non/loosely adherent DCs were used as the cell source for CD11c-isolation and immediately following purification maturation marker (CD80/86) expression were similar to pre-isolation levels (Table 6-2, Fig. 6-2B for C57BL/10 and Fig. 6-3B for C57BL/10ScSn). Simultaneously, the extent of cellular adhesion was calculated by collecting and counting both adherent and non/loosely adherent fractions throughout the culture time, and it was found that at least 40% of cells were adherent to

TCPS substrate during this culture period (Fig. 6-4).

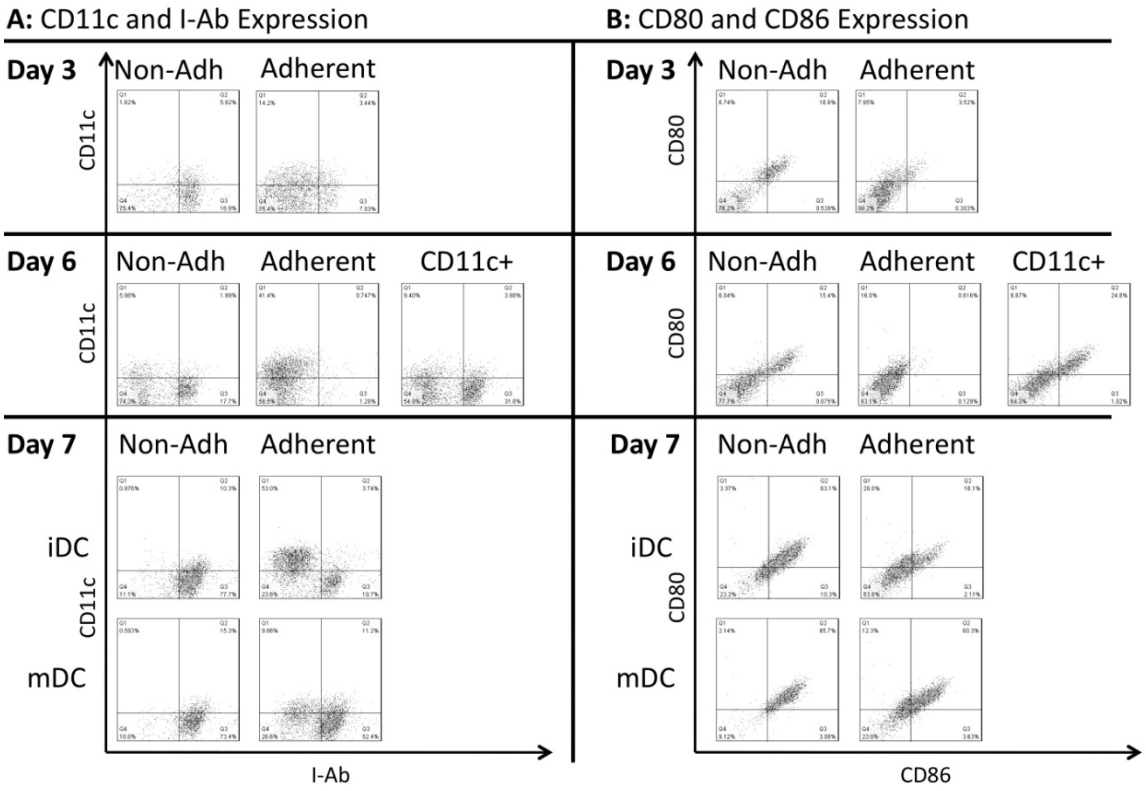

Figure 6-2: Dot plots of gated DC population showing expression levels of (A) CD11c/I-A^b or (B) CD80/CD86 for BMDCs from C57BL/10 over the culture conditions. Non/loosely adherent (Non-Adh) and adherent BMDCs were analyzed separately on Day 3, Day 6 (before and after CD11c-isolation, CD11c$^+$) and on Day 7 following treatment of CD11c$^+$ isolated BMDCs with ultrapure-LPS for 24h. Quadrants drawn were used to show relative levels across the culture conditions. Representative images from one of three independent experiments. Similar data for C57BL/10ScSn mouse strain can be found in the Fig. 6-3.

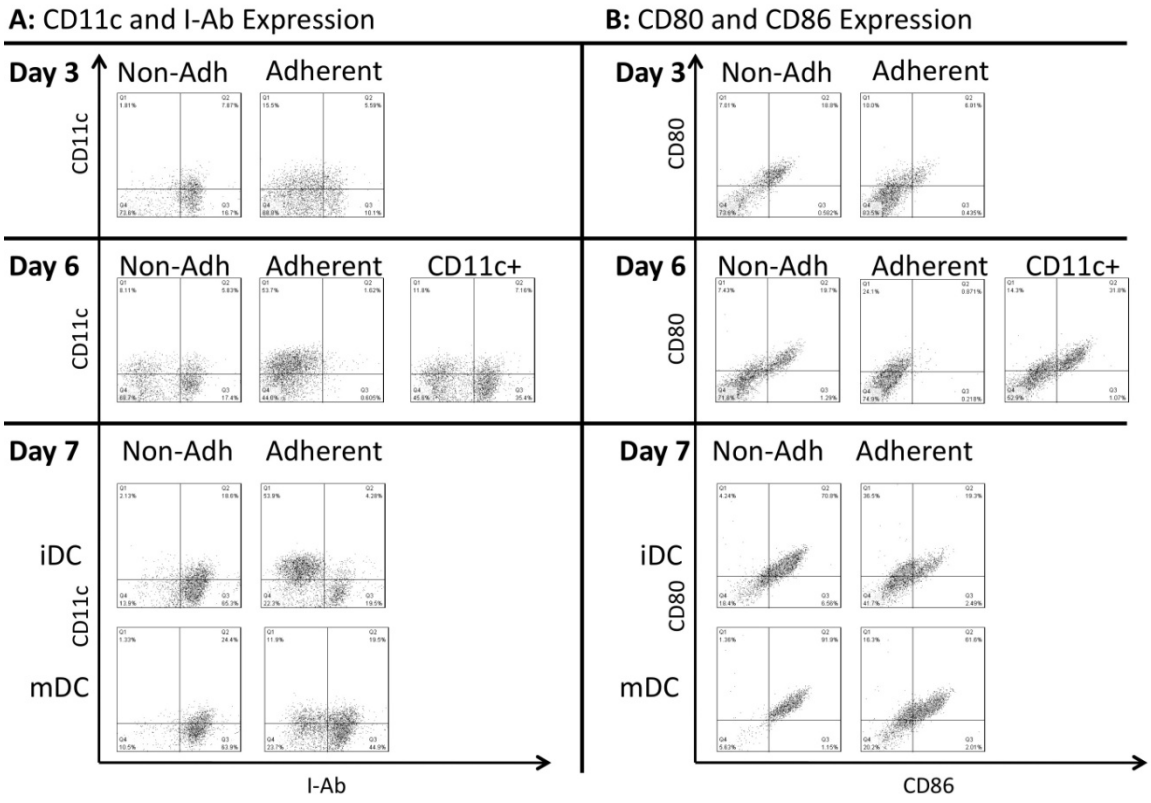

Figure 6-3: Dot plots of gated DC population showing expression levels of (A) CD11c/I-A^b or (B) CD80/CD86 for BMDCs from C57BL/10ScSn over the culture conditions. Non/loosely adherent (Non-Adh) and adherent BMDCs were analyzed separately on Day 3, Day 6 (before after CD11c-isolation, CD11c$^+$) and on Day 7 following treatment of CD11c$^+$ isolated BMDCs with ultrapure-LPS for 24h. Quadrants drawn were used to show relative levels across the culture conditions. Representative images from one of three independent experiments.

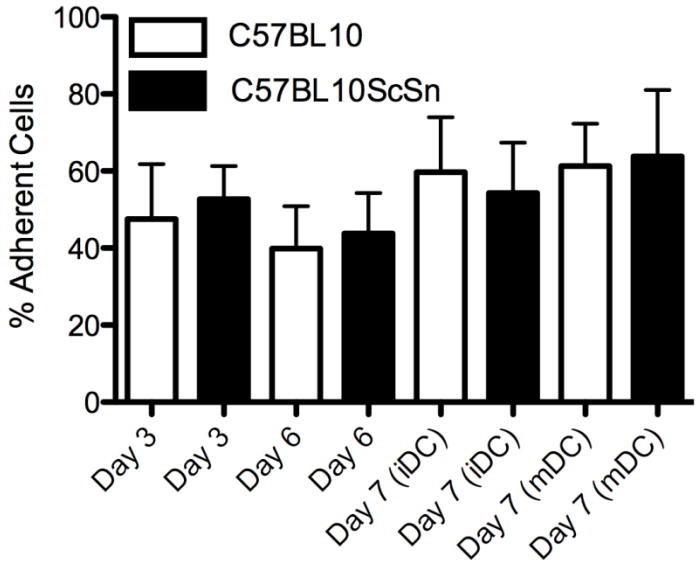

Figure 6-4: Percentage of adherent BMDCs across culture time frame. Non/loosely adherent and adherent DCs were collected and counted using Coulter Counter (10-20μm) at Day 3, Day 6 and on Day 7 following treatment with LPS (mDC) or left untreated (iDC) for C57BL/10 (clear bars) and C57BL/10ScSn (filled bars). Bars show mean+s.d, n=3.

Adherent and Non/loosely Adherent DC Response to LPS Treatment

The responsiveness of Day 6 plated, $CD11c^+$ BMDCs, derived from C57BL/10 or C57BL/10ScSn mice, to the maturation stimulus, LPS, following a 24h treatment, was assessed. It was found that cultures of iDC derived from C57BL/10 or C57BL/10ScSn mice contained adherent BMDCs possessing higher levels of CD11c expression and decreased levels of $I\text{-}A^b$ expression (Fig. 6-2A or Fig. 6-3A, respectively) which also exhibited lower levels of both CD80 and CD86 (Fig. 6-2B or Fig. 6-3B, respectively) than their non/loosely adherent DC counterparts. Particularly for iDCs from C57BL/10, adherent DCs were more responsive to LPS as increases in both $I\text{-}A^b$ and CD86 for adherent cells were significantly higher than non/loosely adherent cells (Fig. 6-5A). For

iDCs from C57Bl/10ScSn mice, only I-A^b expression showed significantly higher increase in expression for adherent cells as compared to non/loosely adherent DCs in response to LPS (Fig. 6-5B). For iDCs derived from either strain, expression of CD11c (α_X integrin) increased in response to LPS for non/loosely adherent DCs but decreased significantly for adherent DCs (Fig. 6-5). Extent of DC adhesion was unaffected by LPS treatment for both strains (Fig. 6-4).

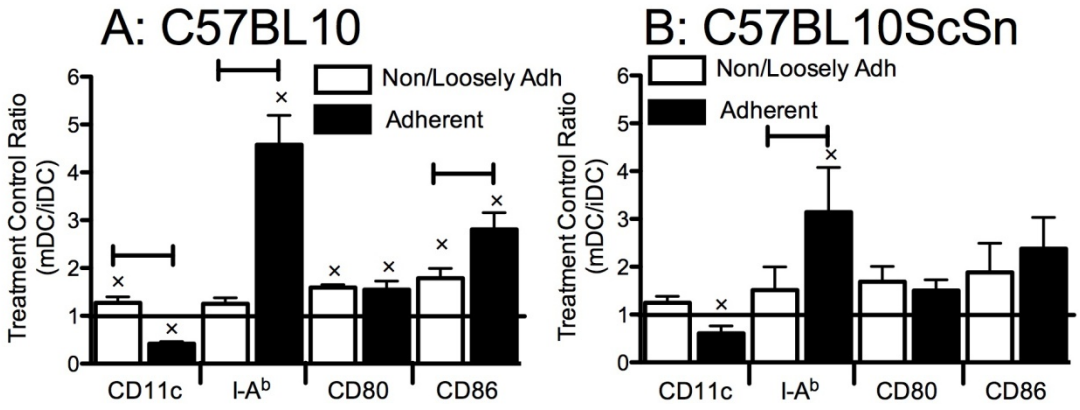

Figure 6-5: Treatment control ratios (mDC/iDC) of either non/loosely adherent or adherent BMDCs for CD11c, I-A^b, CD80 and CD86 expression from (A) C57BL/10 or (B) C57BL/10ScSn. gMFIs were determined for ultrapure-LPS treatment (mDC) of BMDCs for non/loosely adherent or adherent BMDC fractions and divided by levels for untreated non/loosely adherent or adherent BMDCs fractions of iDC controls, respectively. Clear bars, non/loosely adherent DCs; filled bars, adherent DCs. Bars show mean+s.d. X indicates statistical difference from levels of iDC (1), Student t-test, $p \leq 0.05$. Brackets indicate statistical difference between response of non/loosely adherent and adherent BMDCs within the same strain (ANOVA, $p<0.05$), n=3 independent determinations.

Adherent and Non/loosely Adherent DC Response to Biomaterial Treatment

Previously, non/loosely adherent DCs derived from BM of C57BL/6 mice were found to respond to 24h biomaterial treatments of PLGA (Yoshida et al. 2007) . Since adherent DCs appeared to be more responsive to stimuli and biomaterials may be more of a moderate stimulus for DC maturation than TLR ligands, both the adherent and non/loosely adherent BMDC fractions were examined for their responsiveness to biomaterial treatments. Adherent BMDCs from C57BL/10 or C57BL/6 mice were more responsive to LPS treatment by showing greater increases in I-A^b and CD86 expression than their non/loosely adherent DCs counterparts (Fig. 6-6A,B or 6-6D,E, respectively). The response of adherent DCs to PLGA films was also more pronounced in comparison to non/loosely adherent DCs when examining I-A^b expression for C57BL/10-derived BMDCs (Fig. 6-6A,B) and CD86 expression on C57BL/6-derived BMDCs (Fig. 6-6D,E). While non/loosely adherent BMDCs from C57BL/6 mice did show a slight (but significant) increase in I-A^b in response to PLGA films, the increase observed for the adherent DCs was higher but not statistically different from level of iDC (Fig. 6-6D,E).

Adherent BMDCs from C57BL/10 showed a significantly lowered expression of CD11c in response to treatment with PLGA films or MPs (Fig. 6-6B) which was not observed for adherent BMDCs derived from C57BL/6 mice (Fig. 6-6E). Also, PLGA MPs induced no significant alterations in expression of the DC maturation markers investigated (CD80, CD86, I-A^b) on either cell fraction from C57BL/6 mice but did induce a lower expression level for several maturation markers (I-A^b, CD86) in the adherent BMDCs from C57BL/10 mice (Fig. 6-6). Lastly, levels of TNF-α secretion into culture supernatants in response to BMDC treatment with LPS or biomaterials were determined. For BMDCs derived from C57BL/10 or C57BL/6 strains, while treatment with LPS induced high levels of TNF-α secretion, treatment with PLGA films induced slight, but significant increases in TNF-α levels (Fig. 6-6C or Fig. 6-6F, respectively). Treatment of

BMDCs from either mouse strain with PLGA MPs did not induce a significant TNF-α secretion (Fig. 6-6C,F).

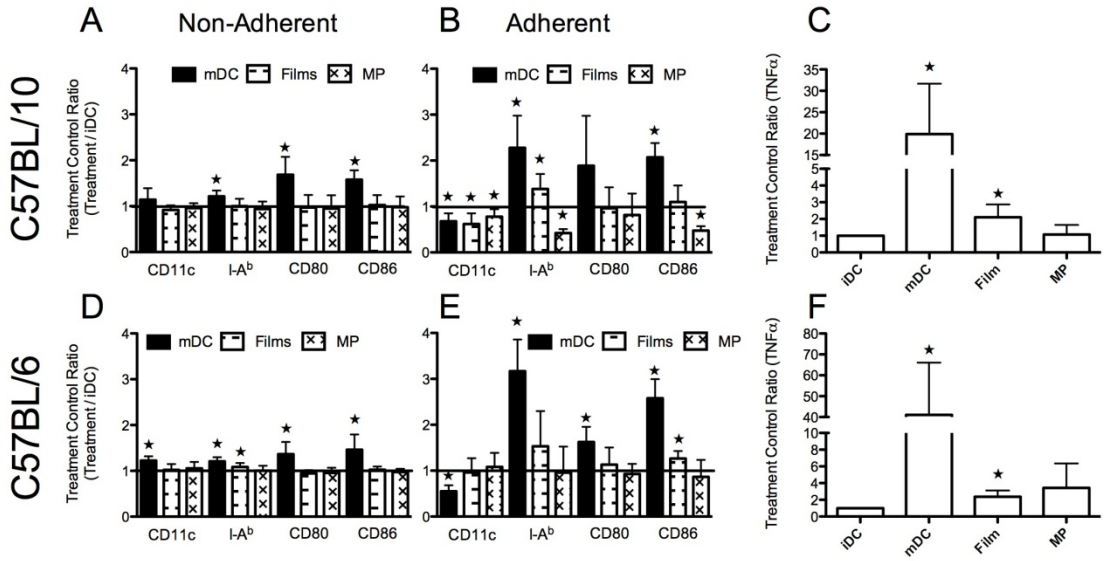

Figure 6-6: Influence of biomaterial treatments on non/loosely adherent and adherent BMDCs. Treatment control ratios (Treatment/iDC) of non/loosely adherent or adherent BMDCs for CD11c, I-A^b, CD80 and CD86 expression from (A-B) C57BL/10 or (D-E) C57BL/6. gMFIs were determined following treatment with ultrapure-LPS (mDC), PLGA film (Films) or PLGA MPs (MP) of BMDCs for non/loosely adherent or adherent BMDC fractions and divided by levels for untreated non/loosely adherent or adherent BMDCs fractions of iDC controls. TNF-α concentration in supernatants of BMDCs from (C) C57BL/10 or (F) C57BL/6 was also determined for iDC, mDC or PLGA film or MP treatments, divided by total cells (both adherent and non/loosely adherent) collected and normalized to the levels of iDC control (1). Star indicates statistical difference from levels of iDC (1), Student t-test, $p \leq 0.05$. Bars show mean+s.d, n=7-8 independent determinations for both strains.

Discussion

Routinely, the non/loosely adherent fraction of murine BMDC cultures is used for analysis, wherein the adherent BMDC fraction is unused. However, here we have shown that adherent murine BMDCs may in fact be of more immature phenotype with more responsiveness to both pathogen and biomaterial stimuli potentially making them the more relevant BMDC fraction to study. Adherent BMDCs from C57BL/10 or C57BL/10ScSn mouse strains expressed higher levels of CD11c, a mouse DC marker, and lower levels of MHC class II as well as CD80/86 than their non/loosely adherent counterpart (Fig. 6-2 and Fig. 6-3, respectively). Upon stimulation with LPS, these molecules' surface presence became relatively higher in the adherent DC fraction from three different mice strains than in the non/loosely adherent fraction (Fig. 6-5 and Fig. 6-6). Also, biomaterial (PLGA) stimulation induced higher increases in surface expression of CD86 and MHC class II molecule for adherent BMDCs in comparison to their non/loosely adherent BMDCs counterparts (Fig. 6-6).

On Day 0 of culture, little to no presence of cDCs was found in BM by examining CD11c expression. This agrees with the findings of others who have shown that only about 0.5% of BM cells are $CD11c^+$ (Diao et al. 2004). The BM from C57BL/10 or C57BL/10ScSn mice stained positive to varying degrees for granulocytes (Gr-1), B cells (or B cell precursors) (CD19), myeloid/macrophage marker (F4/80), and MHC class II ($I\text{-}A^b$) (Table 6-2). The percentages of positive expression observed for Gr-1 and F4/80 (Table 6-2) are in agreement with previous findings for BM cells (Li et al. 2004), and while Gr-1 can be present on monocytes during development (Auffray et al. 2009), BM cells were negative for CD14 (Table 6-2). It is believed that DCs are derived from MHC class II-negative precursors (Inaba et al. 1993; del Hoyo et al. 2002), and here the majority (69-77%) of BM cells were indeed negative for $I\text{-}A^b$ (Table 6-2). There was also little to no positive staining for markers for other cell types including CD3, TER119,

PDCA-1, CD49b, CD117, CD121a and CD138 on Day 0 throughout the culture time frame. This is in agreement other BM cell characterization studies examining CD3, CD14 and CD117 expression (Diao et al. 2004; Menges et al. 2005; Peche et al. 2005).

By Day 3 of BMDC culture, $CD11c^+$ DCs had developed and differential levels of DC marker expression began to appear between the adherent and non/loosely adherent DC fractions for BMDCs from either mouse strain investigated (C57BL/10 or C57BL/10ScSn). While adherent BMDCs expressed primarily mid/high levels of CD11c and low levels of $I\text{-}A^b$, CD80 and CD86, non/loosely adherent BMDC expressed low levels of CD11c and higher levels of $I\text{-}A^b$, CD80 and CD86 (Table 6-2, Fig. 6-2, and Fig. 6-3). Furthermore, by Day 6, the differences between adherent and non/loosely adherent BMDCs were more pronounced especially for the surface presence of CD11c and $I\text{-}A^b$ (Fig. 6-2A, and Fig. 6-3A). The $CD11c^+$ population of the non/loosely adherent DC population was then isolated via positive selection using magnetic beads on Day 6. This isolation did not seem to have an immediate effect on the DC maturation marker expression (Fig. 6-2 and Fig. 6-3). This implies the purification technique did not (at least immediately) activate BMDCs. However, the impact of the CD11c-isolation procedure was not fully characterized in this study, but may offer an explanation as to why the non/loosely adherent BMDC population was not as responsive to stimuli in comparison to others' (Agger et al. 2000). In future studies, if using a CD11c-isolation procedure, it may be beneficial for the purification to utilize solely adherent BMDCs (or at least pooled adherent and non/loosely adherent BMDCs) on Day 6, as adherent BMDCs expressed higher levels of CD11c. Also, the non/loosely adherent cell population was only about 40-50% of the DCs while the adherent fraction was composed of a purer DC population (about 70-80% DCs) as determined by CD11c expression and FSC/SSC combined data (data not shown) for C57BL/10 or C57BL/10ScSn mouse strains. However, adherent BMDCs would likely be more susceptible to activation during isolation due to their

immature state which may make this modification counter-productive.

Following isolation on Day 6, CD11c-isolated DCs were re-plated and treated with LPS for 24h to investigate the responses of both adherent and non/loosely adherent iDCs to TLR ligand stimulation. The adherent fraction of iDC controls from either mouse strain investigated once again were of a more immature phenotype while the non/loosely adherent fraction showed higher expression levels of DC maturation markers and lower expression levels of CD11c (Fig. 6-2 and Fig. 6-3). In fact, the adherent fraction was composed mostly of an I-A^b-low population of DCs. This is in agreement with other studies that have shown that MHC class II^{low} DCs are more adherent than MHC class II^{hi} DCs (Masurier et al. 1999). Furthermore, while the non/loosely adherent fraction was responsive to LPS as far as all markers assessed, the adherent fraction was significantly more responsive especially seen through an increase in I-A^b surface expression (Fig. 6-5). This is in contrast to Peche et al who have shown adherent BMDCs to be more immature but less-responsive to LPS stimulation than non/loosely adherent BMDCs (Peche et al. 2005). The difference in response here may be due to the treatment and analysis of adherent and non/loosely adherent BMDCs fractions combined versus the separation and re-plating of adherent and non/loosely adherent BMDCs prior to treatment performed by Peche et al. Paracrine and direct cellular interactions between non/loosely adherent iDCs and adherent iDC in the work presented here may aid in maintaining the responsive phenotype of the adherent iDC population.

The adherent fraction of BMDCs exhibited a significantly lower level of CD11c expression in response to LPS in contrast to non/loosely adherent DCs which exhibited an increase across the three strains investigated (Fig. 6-5 and Fig. 6-6). The non/loosely adherent BMDC's increase in CD11c expression in response to LPS has previously been seen (Pendl et al. 2002); however, decreased CD11c response following LPS stimulation for the adherent BMDC population has not. Interestingly, these shifts in

integrin expression occur in the absence of any significant alteration in DC adhesion (Fig. 6-4). The adherent fraction possibly has higher basal level CD11c expression due to adhesion to underlying substrate, which is clearly limited in the non/loosely adherent cell population. While higher expression of an integrin (CD11b) negatively correlated to ovine BMDC responsiveness to TLR stimuli (Foulon and Foucras 2008), here, adherent BMDCs with increased integrin (CD11c) expression displayed increased sensitivity to stimulation with LPS (Fig. 6-2 & Fig. 6-5). Others have found that adherent BMDCs expressed lower levels of CD11c than their non/loosely adherent BMDC counterpart (Masurier et al. 1999). Some of the discrepancies between these results (which only utilize GM-CSF-induced differentiation) and the findings presented here may be in part to the addition of IL-4 during BMDC development, as IL-4 has been shown to increase expression of CD11c on BMDCs (Masurier et al. 1999). It was previously believed that GM-CSF alone was unable to produce an adherent BMDC population but rather an adherent population similar to macrophages (Inaba et al. 1992). However, recent findings point that GM-CSF alone may be capable of producing adherent and non/loosely adherent BMDCs which are similar across many DC markers (Li and Lu 2010). *In vitro* supplement of IL-4 in BMDC culture conditions used herein, in addition to use of GM-CSF, may modulate the phenotype and responsive of BMDCs by altering their integrin expression and subsequent adhesion properties. Perrot et al found that higher CD11c expression on infiltrating DCs directly purified from tumors correlated with increased responsiveness of DCs to LPS stimulation (Perrot et al. 2007). Thus, adherent BMDCs which expressed high levels of CD11c in the culture conditions presented here in the presence of IL-4, seemed to mimic this infiltrating DC phenotype *in vivo*. It is unknown whether there is direct interaction between CD11c integrin and substrate; however, CD11c does mediate adhesion to denatured proteins in response to 'danger' which may be adsorbed to the surface (Vorup-Jensen et al. 2005). It is possible then that

CD11c engagement may play a role in BMDC adhesion and influence its responsiveness to TLR stimulation.

The adherent and non/loosely adherent BMDC response to biomaterial treatments (PLGA film or PLGA MPs, which have previously been shown to induce DC maturation) was examined using BMDCs from C57BL/10 or C57BL/6 mice. Non/loosely adherent and adherent BMDCs from both strains were responsive to LPS treatment; however, adherent BMDCs from both strains were once again more responsive to LPS than non/loosely adherent BMDCs (Fig. 6-6). Accordingly, the response of adherent BMDCs to PLGA films was higher than non/loosely adherent DCs in either strain as seen in increases in I-A^b expression in C57BL/10 mice and increases in CD86 expression in C57BL/6 mice (Fig. 6-6). Previously, non/loosely adherent BMDCs from C57BL/6 analyzed following BMDC culture on PLGA films had exhibited significant signs of DC maturation as seen through increased CD80 expression (Yoshida et al. 2007), and here PLGA films also induced a slight but significant extent of DC maturation with an increase in I-A^b expression for non/loosely adherent BMDCs from the same strain (Fig. 6-6D). However, analysis of adherent BMDCs from the C57BL/6 mice seemed to show even greater responsiveness to the biomaterial treatment (Fig. 6-6E). Though a potent stimulator of maturation, such as a TLR ligand, may induce increases in maturation marker expression of non/loosely adherent BMDCs, a more moderate maturation stimulus such as a biomaterial (e.g. PLGA), which may only be partially in contact with this non/loosely adherent population, may not induce further maturation due to their more “pre-activated” condition (high MHC class II, CD80 and CD86 expression). However, adherent BMDCs which took on a more “immature” phenotype (low MHC class II, CD80 and CD86 expression), would more likely have the ability to respond to such a biomaterial stimulus and here displayed signs of DC maturation which was mostly absent in the non/loosely adherent BMDC populations (Fig. 6-6). Furthermore, the

increased response to PLGA of adherent BMDCs may be due in part to directly interacting with biomaterial surfaces through adhesion receptors or other pertinent PRRs (Babensee 2008; Shokouhi et al. 2010). Of note, however, is the lack of increase in expression of all DC maturation markers investigated following biomaterial treatment, which was found in response to LPS. As a practical assessment of adherent BMDC maturation, to assure that PLGA films induce functionally stimulatory DCs, biomaterial or LPS treated adherent or non/loosely BMDCs could be collected and utilized in an allogeneic mixed lymphocyte reaction. This would allow for investigation into whether biomaterial treatment produces activated adherent DCs, and also assess whether treated adherent BMDCs are more stimulatory than treated non/loosely adherent BMDCs, as anticipated.

Similar to the response of adherent BMDC treatment with LPS, culture of BMDCs in the presence of PLGA films also induced significant decrease in the expression of CD11c in the adherent BMDC fraction from C57BL/10 mice (but not C57BL/6 mice). Presumably, these strain-dependent differences in responses to PLGA films are due to some genetic differences [e.g. identified at multiple loci on chromosome 4 (McClive et al. 1994)] even though the two strains are genetically related.

PLGA MP treatment of BMDCs, which has previously been found to increase expression of DC maturation markers on human monocyte-derived DCs (Yoshida and Babensee 2004; Yoshida and Babensee 2006), did not induce any significant sign of maturation in adherent or non/loosely adherent fractions of BMDCs from either C57BL/10 or C57BL/6 mice. In fact, adherent BMDCs from C57BL/10 exhibited significant decreases in I-A^b and CD86 expression while adherent BMDCs from C57BL/6 displayed no significant changes in DC maturation marker expression when in contact with PLGA MPs (Fig. 6-6B,E). The lack of DC maturation marker expression level changes effects in response to PLGA MP in both non/loosely adherent and adherent

BMDCs from C57BL/6 (Fig. 6-6D,E) matches the previous response found of BMDC from this strain to PLGA MP (Yoshida et al. 2007). The contrary decrease in level of maturation marker expression on adherent BMDCs from C5BL/10 (as opposed to no alteration in BMDCs from C57BL/6) in response to PLGA MP may also be related to the genetic differences noted between the strains (McClive et al. 1994).

Treatment of BMDCs with LPS induced high levels of pro-inflammatory cytokine (TNF-α) secretion, while their treatment with PLGA films also significantly induced (though to a lesser degree) TNF-α secretion for BMDCs from both C57BL/10 and C57BL/6 mice. These results further illustrate the inflammatory and maturation-inducing effect of both LPS and biomaterial film treatment on BMDCs. PLGA MPs, however, did not induce production of TNF-α from BMDCs of either strain corresponding to the non-inflammatory response exhibited by lack or down-regulation of DC maturation marker expression from adherent BMDCs from C57BL/6 or C57BL/10, respectively. Therefore, the physical form of PLGA may influence the response elicited by BMDCs.

In conclusion, the findings presented here show evidence that during the BMDC derivation in culture under the influence of GM-CSF/IL-4 stimulation, adherent BMDCs remained immature and more responsive to either pathogen or biomaterial stimulus. This is in contrast to non/loosely adherent BMDCs, which appeared to take on a mature phenotype, and while being susceptible to potent TLR ligand stimulation, were not as responsive to biomaterial treatments in PLGA film or MP form. With this it is suggested that the adherent BMDC population be characterized and assessed following BMDC treatment due to their more responsive nature, particularly for more moderate stimulating agents such as biomaterials.

CHAPTER 7

THE ROLE OF INTEGRINS IN THE RECOGNITION AND RESPONSE OF DENDRITIC CELLS TO BIOMATERIALS [*]

Introduction

Dendritic cells are sentinels continuously surveying the body for pathogens. Upon recognition and uptake of foreign entities, their status as professional antigen-presenting cells (Banchereau and Steinman 1998; Steinman and Banchereau 2007) serves to link the innate immune response to an antigen-specific adaptive response. DCs possess PRRs, which aid in their response to foreign entities (e.g. pathogens) by binding pathogen-associated molecular patterns (PAMPs) (Janeway and Medzhitov 2002; Akira et al. 2006). Upon recognizing PAMPs, DCs undergo a phenotypic shift from an immature (iDC) to a mature (mDC) state. As iDCs, they lack T cell stimulatory capacity (with low CD80/CD86 and major histocompatibility complex (MHC) class II surface expression), have a high phagocytic capacity and also have a rounded morphology. But upon maturation, DCs up-regulate CD80/CD86 as well as MHC II expression (Mellman and Steinman 2001; Reis e Sousa 2006; Steinman and Banchereau 2007). Mature DCs also have a low phagocytic capacity and possess a "dendritic" morphology for increased interaction with T cells (Steinman and Banchereau 2007). The other side of maintaining immune homeostasis is that interaction of T cells with iDC or tolerogenic DCs maintains or induces immune tolerance through induction of T cell anergy or regulatory T cell induction (Banchereau and Steinman 1998). Thus, DCs may activate or passivate an immune response accordingly depending on their phenotypic status.

[*] Modified from Rogers TH, Babensee JE "The Role of Integrins in the Recognition and Response of Dendritic Cells to Biomaterials" (*Biomaterials,* 2010. doi:10.1016/j.biomaterials.2010.10.014)

Tissue engineered devices utilize a biomaterial delivery vehicle (e.g. scaffold or a microparticle) for a biological component (Stock and Vacanti 2001; Nerem 2006). The host response to a tissue engineered device is composed of a non-specific inflammatory response toward the biomaterial component and an antigen-specific immune response toward the biologic (Babensee et al. 1998; Babensee 2008). In the case of non-autologous biologics, an immune response toward the therapeutic cell or protein is a major challenge, for example in tissue engineering (Nerem 2007), and we have previously found that the biomaterial scaffold may act as an adjuvant toward the co-delivered biologic (Matzelle and Babensee 2004). Biomaterial scaffolds of PLGA induce significantly higher and longer lasting humoral immune response toward a model antigen, ovalbumin, than scaffolds made of agarose (Norton et al. 2010). We hypothesize that the biomaterial-induced adjuvant effect (or lack thereof) is due to the DC response within the complex non-specific inflammatory reaction toward the biomaterial *in vivo* (Babensee et al. 1998; Babensee 2008). Furthermore, we have found that DCs treated with biomaterials used in combination products *in vitro* differentially induce or inhibit DC maturation (Babensee and Paranjpe 2005; Yoshida and Babensee 2006; Yoshida et al. 2007). PLGA or chitosan films induced maturation of DC as seen in increases in cytokine production and CD86 expression, while this effect was not observed when DCs were treated with hyaluronic acid or agarose films. This illustrates the importance of material selection for tissue-engineered devices or vaccine delivery systems as biomaterials may influence the immune response toward the co-delivered biologic.

To begin to understand biomaterial-induced DC maturation, an investigation was undertaken to understand DC adhesion to biomaterials and its contribution to DC phenotype. This direction was suggested by preliminary observations that indicated an increase in DC adhesion on PLGA compared to other substrates (TCPS or agarose

films). Cellular adhesion to biomaterials has been extensively studied as it mediates cellular responses with beneficial effects (e.g. endothelial coverage on vascular grafts) or detrimental effects (e.g. fibrous encapsulation) (Anderson et al. 2008). The most well-characterized adhesion molecules used by leukocytes to mediate their adhesion to biomaterials are integrins (Springer 1990), which are transmembrane heterodimer receptors composed of both α and β subunits (Plow et al. 2000; Abram and Lowell 2009). Leukocytes uniquely possess the β_2 family of integrins ($\alpha_L\beta_2$, $\alpha_M\beta_2$, $\alpha_X\beta_2$) which allow for transmigration in response to infection or inflammation (Anderson 2001). β_2 integrins have been shown to be involved in the adhesion of monocyte/macrophages to biomaterials (Davis 1992; Annenkov et al. 1996; McNally and Anderson 2002) and phagocyte accumulation on implants *in vivo* (Tang et al. 1996). Circulating or monocyte-derived myeloid DCs, which are particularly relevant in the response to biomaterial implants, possess integrins of both the β_1 and β_2 family (McCarthy et al. 1997; Ammon et al. 2000). DC integrin-mediated adhesion to known ligands such as fibronectin (Swetman Andersen et al. 2006; Kohl et al. 2007) and fibrinogen (Thacker and Retzinger 2008) pre-coated on material surfaces has been shown. Furthermore, while different plasma/serum protein substrates elicit similar DC adhesion, they are capable of differentially influencing cytokine production (Acharya et al. 2008); thus, different protein substrates may influence the response of DCs. As PRRs, $\alpha_M\beta_2$ (complement receptor, CR3) and $\alpha_X\beta_2$ (CR4) play a role in DC recognition of opsonized foreign entities, and CRs have been shown to play a role in monocyte recognition of a biomaterial (McNally and Anderson 1994). However, to date the role of integrin-mediated adhesion in substrate-induced DC maturation remains unknown.

Materials and Methods

Biomaterial Preparation

For more detailed description of biomaterial preparation, see CHAPTER 5. Briefly, 10% (w/v) PLGA (mole ratio 75:25, inherent viscosity 0.70 dL/g in trichloromethane; Durect/Lactel Absorbable Polymers, Birmingham, AL) was prepared (Yoshida and Babensee 2004). PLGA films (2mm in thickness) for use were punched out with arch punches for 24 well plates (9/16'') or 6 well plates (5/4'') and rinsed for 1h with sterile water. Films were allowed to dry in biosafety cabinet, sterilized with UV (30min each side) and rinsed with endotoxin free water immediately prior to use. PLGA films were tested for endotoxin content using Limulus Amebocyte Lysate (LAL) assay (QCL-1000, Lonza, Basel, Switzerland) with detected levels of approximately 0.1 EU/mL. Three percent (w/v) agarose (Type V) (Sigma) films were prepared by microwaving agarose/water suspension for 30s. This solution was then cast in plates and allowed to cool at 4^{o}C for 30 min. The films were brought to room temperature for at least 1h before use in cell culture. Resulting films were distributed across the wells with an average thickness of 3mm (approximately 2mm in the center of well and 4mm at edges).

Human Monocyte-Derived Dendritic Cell Culture

Human peripheral blood was drawn from healthy donors with informed consent at Georgia Tech Student Health Center by trained phlebolotomist in accordance with Georgia Institute of Technology Institutional Review Board (IRB) protocol #H10011. Dendritic cells were differentiated from monocytes as previously described (Romani et al. 1996) with modifications (Yoshida and Babensee 2004). For detailed description of culture, please see methods section of CHAPTER 5 (Human Monocyte-derived DC Examination of Biomaterial Effects).

Dendritic Cell Treatment and RNA Extraction, cDNA Synthesis

Dendritic cells were collected on Day 5 of culture and treated in 6 well plates (Costar) (Corning, Corning, NY) with fresh complete media as untreated iDC for negative control, treated with ultrapure-LPS (a TLR4-specific ligand, 1μg/mL) (InvivoGen, San Diego, CA) as a positive-control for maturation to provide mature DCs (mDCs), or cultured on PLGA or agarose films for 24h at 37°C and 5% CO_2. Cell viability is routinely > 95% following treatment as assessed via propidium iodide staining or trypan blue exclusion. Non-adherent DCs were collected by gentle pipetting and pooled together with adherent DCs which were collected by addition and pipetting with cell dissociation solution (Sigma, St. Louis, MO). This pooled fraction was then purified for DCs using magnetic bead isolation. Briefly, cells were incubated with dendritic-cell specific ICAM grabbing non-integrin (DC-SIGN) microbeads along with Fc-receptor blocking solution (Miltenyi Biotec) per manufacturer's protocol, rinsed and passed through an LS column placed in a midiMACs (Miltenyi Biotec) magnet to purify DC-SIGN$^+$ DCs and remove B cells. To assess purity, DC-SIGN$^+$ fraction was double-stained with DC-SIGN-FITC (R&D Systems, Minneapolis, MN, clone 120507) and CD19-APC (Miltenyi Biotec, clone LT19). Final purified cell population was at least >95% DC-SIGN$^+$ and <1% CD19$^+$ (APPENDIX 2).

Purified DCs were used for RNA isolation using RNeasy Mini kit (Qiagen, Valencia, CA) according to manufacturer's instructions. RNA concentration (A_{260nm}) and quality (A_{260nm}/A_{280nm} > 2) was measured using NanoDrop 1000 (Thermo Scientific, Waltham, MA) and stored at -80°C. The Quantitect RT Kit (Qiagen) was used to prepare cDNA according to the manufacturer. For each DC treatment, 10ng of RNA (per single qRT-PCR reaction) multiplied by the number of reactions needed total was used for cDNA synthesis immediately following genomic DNA removal by DNAse I treatment (Qiagen). Reverse transcription was undertaken at 42°C for 20min in a water bath

followed by immediate placement in 95°C heat-block for 3min to inactivate reverse transcriptase. cDNA was then used for q-RT-PCR.

Primer Selection and Gene Expression Analysis

Integrins, adhesion molecules, integrin-related signaling molecules as well as two housekeeping genes (β-actin and β_2-microglobulin) were selected for primer design partially based on known monocyte-derived DC integrin expression (Ammon et al. 2000). Primers were chosen using Harvard PrimerBank (Wang and Seed 2003) (Table 7-1) and appropriate forward and reverse pairs were chosen which minimized hairpin and primer-dimer formation as assessed using NetPrimer (Premier Biosoft International, Palo Alto, CA), were less than or equal to 250bp in amplicon size and had a melting temperature (T_m) of approximately 60°C. Forward and reverse primer synthesis was outsourced (Sigma) and pairs pooled. For validation/sequencing, primer pairs (1μM final concentration) were used to amplify an iDC sample cDNA prepared using HotStarTaq Plus PCR kit (Qiagen) following manufacturer's instructions. Amplicons were shipped overnight to Eurofins MWG Operon (Huntsville, AL) and sequences were validated using Basic Local Alignments Search Tool (BLAST) (Altschul et al. 1990).

Following validation, primer pairs were then used for experimental analysis of relative gene expression. QuantiFast SYBR-Green PCR kit (Siegen) was used to measure PCR amplification of converted cDNA from each treatment via StepOne Plus RT-PCR system (Applied Biosystems, Carlsbad, CA). Briefly, cDNA from each treatment from a single donor was mixed with SYBR-green master mix and aliquoted into a single MicroAmp Fast Optical 96 well plate (Applied Biosystems) along with primer pairs (1μM). An initial activation step was performed at 95°C for 5 min followed 40 cycles of RT-PCR at 95°C for 10s and 58°C for 30s. Melt curve analysis was performed automatically following RT-PCR, and each gene product exhibited single-peak T_m's. Baseline and

threshold level were set manually, and settings used were constant for each sample analyzed.

Threshold cycles (C_t) for each gene were determined and normalized to the average C_t of β-actin and β_2-microglobulin (ΔC_t) across treatments (which did not significantly change). Inter-donor comparison of up or down-regulation of gene expression was determined by comparing ΔC_t values from treated DCs for a specific gene with that of iDCs.

Hierarchical cluster analysis was performed using Matlab (The Mathworks, Natick, MA) to perform a 2D clustergram using $-\Delta C_t$ data. Cluster analysis was performed using a Pearson correlation distance calculation for both genes and treatments. Heat maps were generated in Matlab but bin size and colors were manually chosen.

Table 7-1: Primer pairs for integrins, adhesion molecules and related signaling molecules as well as house keeping genes. Primer pairs were chosen from Harvard Primer Bank (Wang and Seed 2003) with PrimerBank ID listed in table. For detailed information/sequences on each primer pair, please see http://pga.mgh.harvard.edu/primerbank/.

Gene	PrimerBank ID
ITGB1 (CD29)	19743813a3
ITGB2 (CD18)	4557886a1
ITGAL (CD11a)	4504757a2
ITGAM (CD11b)	4504759a3
ITGAX (CD11c)	34452173a3
ITGA4 (CD49d)	4504749a3
ITGA5 (Cd49e)	4504751a1
CD54 (ICAM1)	4557878a2
CD44	21361193a2
FAK	23273417a3
Paxillin	4506345a3
Talin1	16753233a2
Vinculin	4507877a1
Beta Actin	4501885a1
B2M	4757826a1

Intracellular/Extracellular Immunofluorescence of Adherent DCs

Extracellular staining of adherent DCs was completed following culture of human monocyte-derived DCs in 24 well plates on TCPS surface or PLGA films. For noted time points, non/loosely-adherent cells were removed by aspiration and adherent cells were rinsed twice with cold PBS. Cells were then fixed in 4% paraformaldehyde in PBS (Polysciences Inc, Warrington, PA) for 10min and rinsed three times with PBS. Non-specific sites were blocked with 3% human serum albumin (HSA) (EMD Chemicals, Darmstadt, Germany) in PBS at room temperature for 1h. A fluorochrome conjugated antibody for DC-SIGN-(FITC) (R&D Systems, clone 120507) was diluted in 3% HSA, added to wells and incubated for 2h at room temperature. Surfaces were rinsed three times in cold PBS and immediately mounted and coverslip added directly onto sample using ProLong Gold Antifade kit with DAPI (Invitrogen). Bright field and fluorescent images were taken using Nikon Eclipse Ti microscope (Nikon, Melville, NY).

Early (1.5h) DC integrin (extracellular) and F-actin (intracellular) co-localization was monitored with immunofluoresence following DC treatment with PLGA films as described above with the following modifications. DCs were treated with PLGA films in 24 well plates for 1.5hr, rinsed with PBS, and fixed with 4% paraformaldehyde in PBS for 5min. Cells were permeabilized with 0.5% Triton X-100 (Sigma) for 10min, rinsed, and non-specific sites blocked with 5% FBS in PBS for 1hr, 37^{o}C. Subsequently, cells were stained with either monoclonal β_1-FITC (TS2/16, BioLegend, San Diego, CA) or monoclonal β_2-FITC antibodies (TS2/18, BioLegend) in combination with phalloidin-TRITC (Sigma) to visualize F-actin for 1h, 37^{o}C in 5% FBS. Surfaces were then rinsed, and PLGA films were mounted on glass slides and images taken with Zeiss LSM 510 UV confocal microscope (Zeiss, Thornwood, NY).

Integrin Antibody-Blocking Experiments

To undertake integrin antibody blocking, biomaterial treatments of DCs took place in 24 well plates (Corning Costar). Human DCs were cultured as before up to Day 5 and collected and pre-treated with anti-integrin antibodies for 1h at 37°C prior to treatment on either TCPS or PLGA films for 24h. Monoclonal integrin blocking antibodies included: anti-β_1 (Clone: P5D2; Abcam, Cambridge, MA), anti-β_2 (Clone: TS1/18; BioLegend), anti-α_M (Clone: 44 or CBRM1/5; BioLegend), anti-α_L (Clone: HI111; BioLegend), anti-α_X (Clone: 3.9; BioLegend), or isotype mouse IgG1,κ (BioLegend). All antibodies came purified and endotoxin-free except for anti-β_1 which was purified using Protein G column (Pierce) and dialyzed in sterile D-PBS for 2h to remove contaminants. Anti-β_1 and anti-β_2 pre-treatment on TCPS or PLGA were initially tested at increasing concentrations ranging from 10-120μg/mL to determine appropriate blocking concentration. β_1 antibodies were used then at 10μg/mL while anti-β_2 antibodies were used at 10, 20 or 40 μg/mL, as noted in text.

The effect of integrin blocking on DC adhesion was first assessed microscopically. Images were taken of DCs at 3 and 24h on TCPS and PLGA substrates using an Axiovert 135 (Zeiss). Also, the effect of integrin blocking was assessed via cell counting of non/loosely adherent DCs. The number of adherent DCs was not directly assessed because adhesion to TCPS was, for some donors, extremely limited, yielding difficult analysis of adherent cell counts. At 24h, non/loosely-adherent DCs were collected by rocking and gentle pipetting of DCs off the surface. Non/loosely adherent DCs were counted using Multisizer 3 Coulter Counter (Beckman Coulter, Brea, CA) analyzing population between 10-20μm in diameter only. These DCs were pelleted by centrifugation at 400g for 10min while the remaining adherent DCs were collected using addition of cell dissociation solution (Sigma), pipetting and incubation at 37°C to

remove DC from the surfaces. Adherent and non/loosely adherent DCs were pooled together and stained with anti-CD86-PE (Ancell, Minneapolis, MN) for 1h, 4^{o}C to assess DC maturation. DCs were also double stained when necessary with CD86-PE/β_2-FITC (BioLegend) or CD86-PE/β_1-FITC (BioLegend) to indirectly test the binding of anti-β_2 or anti-β_1 blocking antibodies as well as level of maturation with CD86. Flow cytometry was then performed, and DC population gated on the FSC/SSC plot for analysis of CD86 expression. For TCPS-cultured DCs, CD86 expression was defined as "low" (CD86^{low} gate) if CD86 levels were below an intensity of 50. For PLGA-treated DCs, using the same setting for flow cytometry, since CD86 expression increases on the activating biomaterial PLGA, the CD86^{low} gate was doubled to include any cell with intensity below 100. The percentage of total DCs in the CD86^{low} gate (defined as "% immature DCs") was then normalized for each pre-treatment to the levels found on isotype.

Integrin Cross-linking to Biomaterial Surface

To demonstrate direct integrin engagement at the DC-biomaterial interface, a cellular-substrate cross-link was performed and integrins visualized (Keselowsky and Garcia 2005). Specifically, DCs were cross-linked to PLGA using an amine reactive cross-linker with a 12Å spacer arm length, DTSSP (3,3´- Dithiobis [sulfosuccinimidylpropionate]) (Pierce, Rockford, IL). For this method, DCs were treated with PLGA films in 24 well plates for 1.5h and subsequently non-adherent cells were removed via D-PBS rinses. Adherent DCs were then cross-linked to PLGA surface via 1mM DTSSP in 2mM glucose in D-PBS (30 min, room temperature). Unreacted DTSSP was then quenched with 50mM Tris (Sigma) in D-PBS (15min). Non-cross-linked cellular material was then extracted in 0.1% sodium dodecyl sulfate (SDS, Sigma) with 350μg/mL phenylmethylsulfonyl fluoride (Sigma) in D-PBS. Surfaces were then rinsed with D-PBS, and blocked with 5% FBS in PBS for 1h, 37^{o}C. For this experiment only,

polyclonal goat anti-human antibodies against β_1 and β_2 integrins (both from R&D Systems, Minneapolis, MN) were incubated at 1:400 dilution in 5% FBS for 1h, 37^{o}C. Following three D-PBS rinses, surfaces were incubated with donkey anti-goat-FITC secondary antibody (kindly provided by Dr. Robert Nerem of Georgia Institute of Technology) at 10μg/mL (Invitrogen) in 5% FBS for 1h, 37^{o}C. Surfaces were rinsed, mounted as previously described and surfaces visualized with LSM 510 UV confocal microscope (Zeiss). Non-cross-linked controls were also performed as negative controls and yielded no positive integrin staining, indicating cross-linking was necessary to detect integrins engaged by the material surface.

Statistical Analysis

For statistical analysis of DC adhesion and maturation data, an ANOVA analysis was performed (GraphPad, LaJolla, CA) using Tukey-post test ($p<0.05$) with donors nested within treatment ($n=5-8$). When comparing data to a normalized value (1), a two-sided Student t-test was performed ($p\leq0.05$).

Results

To initially investigate the level of DC adhesion to biomaterials, the adherent cell fraction on either TCPS or PLGA surfaces was imaged using microscopy. Adherent cells on both substrates were fixed and stained with DC surface marker (DC-SIGN) to assure the specific assessment of DCs. As shown in Figure 7-1, DCs present on TCPS surface mostly appeared round in morphology (Fig. 7-1A). This is in contrast to DCs treated with PLGA films, which showed more cellular adhesion to the surface and nearly all DCs exhibited dendrites reaching over 100μm (Fig. 7-1B). On both substrates, each adherent cell was DC-SIGN^{+} (a human DC marker) (Fig. 7-1C,D) while being negative CD19 (B cells) (APPENDIX 6, Fig, A6-1), a contaminating cell population in this culture system.

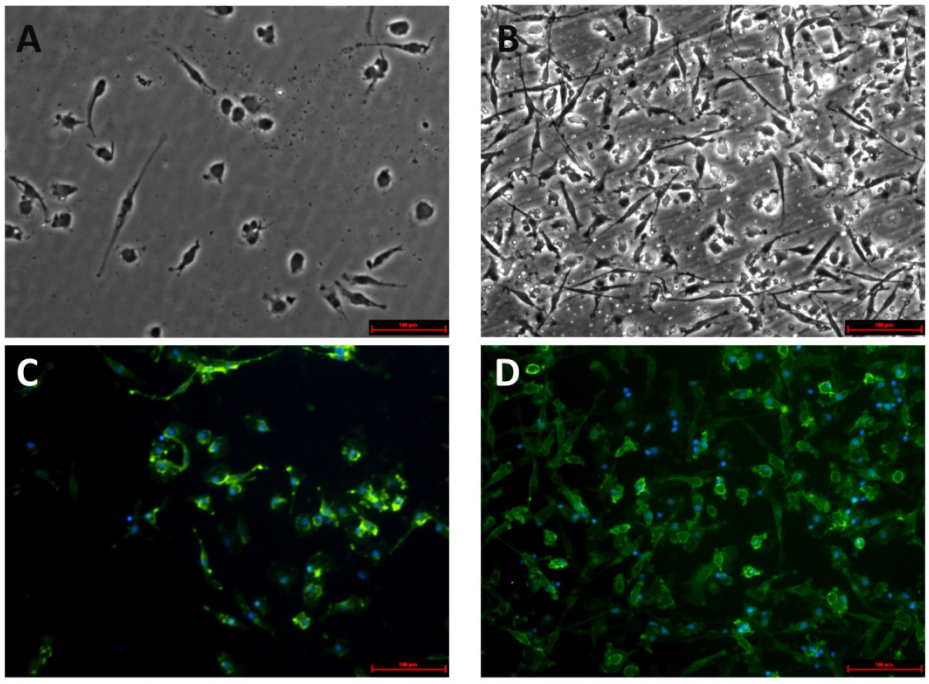

Figure 7-1: Adherent DC morphology on TCPS or PLGA films. DCs were cultured on TCPS (A,C) or treated with PLGA films (B,D) for 24h, stained with DC-SIGN-FITC and DAPI for nuclei visualization (representative of n=3). Microscopy was performed at 20X. Scale bar denotes 100μm.

In order to pinpoint adhesion molecules potentially responsible for the recognition of biomaterials by DCs, a gene expression analysis was undertaken to determine how DC adhesion and/or DC activation influences integrin and other adhesion molecule mRNA expression (chemokine receptor expression was also examined simultaneously, with results in APPENDIX 4). Dendritic cells were cultured on TCPS as a negative control for iDC, treated with PLGA or agarose films (Babensee and Paranjpe 2005; Yoshida and Babensee 2006), or cultured on TCPS and stimulated with ultrapure-LPS as a positive control for mDCs. As shown in Figure 7-2, DCs cultured on TCPS (iDC) or treated with agarose films showed similar gene expression patterns (qualitative analysis as seen through tight cluster) across all adhesion-related molecules investigated. In contrast, the activating treatments of PLGA films or ultrapure-LPS (mDC) clustered together, though to a lesser extent than agarose and TCPS (Fig. 7-2). Adhesion-related signaling molecules (such as FAK) were down-regulated in response to ultrapure-LPS but unaffected upon PLGA treatment. There was a noted difference in the integrin gene expression pattern to PLGA and ultrapure-LPS. While ultrapure-LPS induced down-regulation of integrin subunits (α_M, β_1, β_2, α_X, and α_5), PLGA treatment induced an up-regulation of these molecules in DCs.

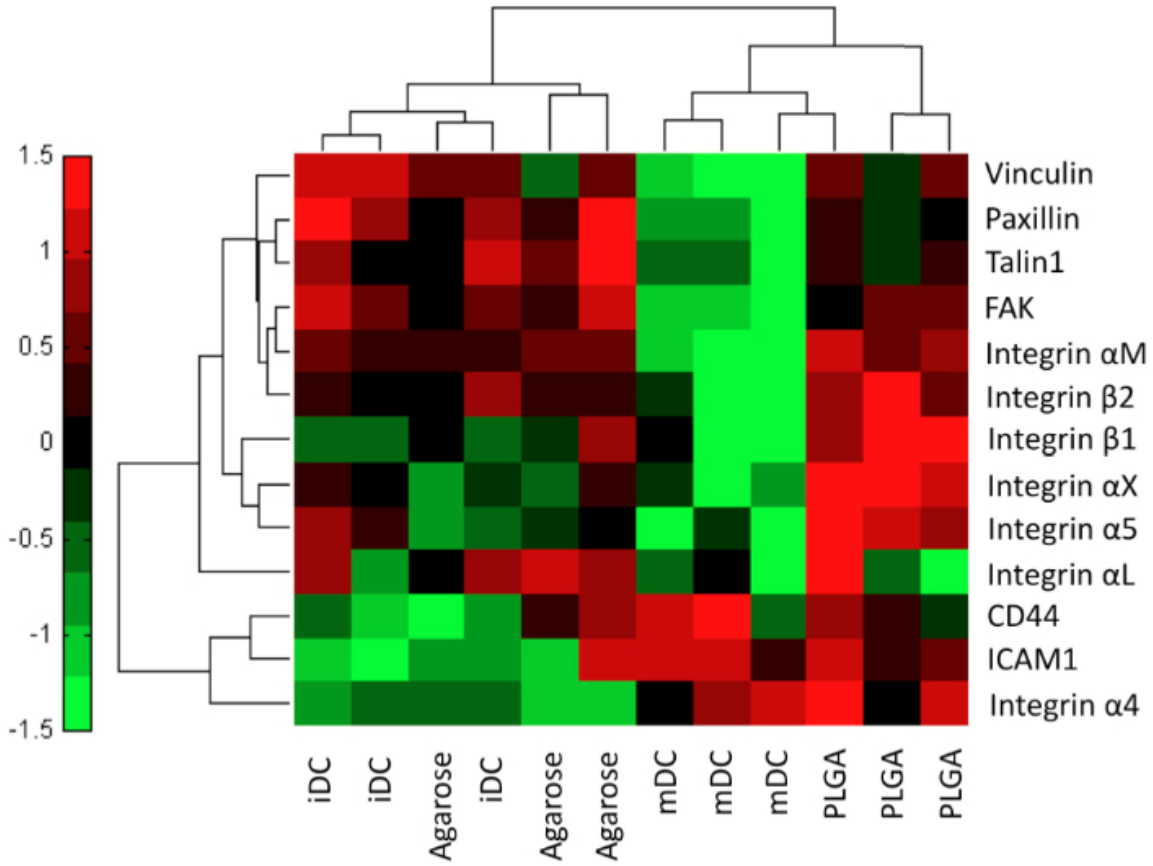

Figure 7-2: Hierarchical cluster analysis of RT-PCR ($-\Delta C_t$) data for integrins and adhesion-related signaling molecules in DCs cultured on TCPS (iDC), treated with ultrapure-LPS (mDC), or treated with PLGA or agarose films for 24h. Heat map and 2D cluster analysis were performed using Matlab *clustergram* function assuming Pearson correlation distance calculation for both genes and treatments. Scale bar and color values denote ±1.5 standard deviation for each gene (row), n=3.

Next, the role of integrin families was investigated in DC adhesion to biomaterials using antibody-blocking techniques to inhibit β_1 and β_2 integrin family members. DC adhesion was measured by assessing the number of non/loosely adherent DCs (an inverse measurement of the number of cells adhering to the substrates) and compared to isotype control treatments. An anti-β_1 blocking antibody (clone P5D2) was unable to affect extent of DC adhesion to both serum-coated (from the culture medium) TCPS or PLGA (Fig. 7-3A) but was able to inhibit DC adhesion and spreading to plasma fibronectin (pFN) coated glass cover slips (Fig. 7-4) which has been shown by others (Swetman Andersen et al. 2006), demonstrating functionality of the mAb with this ligand pre-coating. Two other monoclonal anti-β_1 blocking antibodies (clones AIIB2 and JB1A) were similarly unable to inhibit DC adhesion to TCPS or PLGA (data not shown). Anti-β_2 pre-treatment using blocking mAb TS1/18 (Beals et al. 2001), however, at all concentrations investigated (10, 20 and 40μg/mL) was able to inhibit adhesion of DCs to both TCPS and PLGA films (Fig. 7-3B,C) to levels significantly above isotype controls. The binding ability of anti-β_2 was confirmed via flow cytometry using a fluorescently labeled antibody of the same clone which was unable to bind to DCs when pre-treated with anti-β_2 but successfully bound in the presence of isotype (Figure 7-5). The morphology of DCs treated with PLGA films was altered from being adherent and spread (Fig. 7-3D) to being rounded (Fig. 7-3E) when β_2 binding was inhibited.

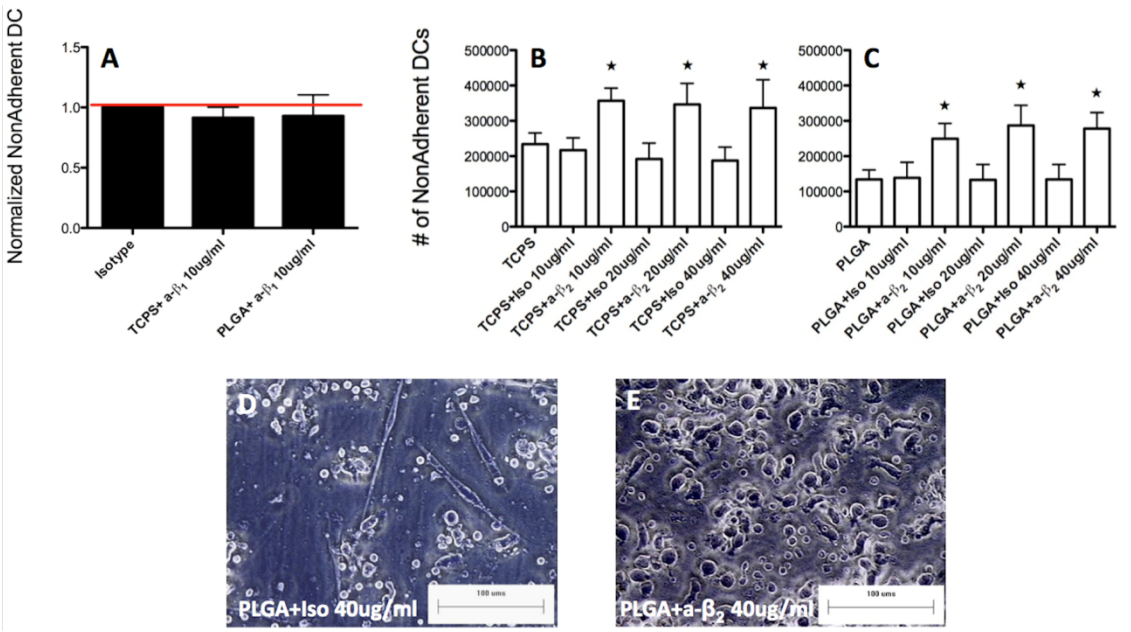

Figure 7-3: β_1 or β_2 integrin antibody blocking of DCs cultured on TCPS or treated with PLGA films for 24h. (A) Anti-β_1 (or isotype) at 10μg/mL treatment was performed and DCs were subsequently on cultured on TCPS or treated with PLGA films for 24h. Non/loosely adherent DCs were collected and counted and values were normalized to that of isotype control (n=2 independent determinations, mean+range). (B) DCs were cultured on TCPS for 24h following anti-β_2 (or isotype) at 10, 20 or 40μg/mL treatment. Non-adherent DCs were collected, counted and averaged. Star indicates statistical difference from respective isotype and TCPS control (ANOVA, $p<0.05$) (n=5 independent determinations, mean+s.d.) (C) DCs were cultured on PLGA for 24h following anti-β_2 (or isotype) at 10, 20 or 40μg/mL treatment. Non-adherent DCs were collected, counted and averaged. Star indicates statistical difference from respective isotype and PLGA control (ANOVA, $p<0.05$) (n=5 independent determinations, mean+s.d.) (D&E) Morphology of DCs cultured on PLGA following isotype (D) or anti-β_2 (E) treatment, representative of n=5 determinations. Scale bar denotes 100μm.

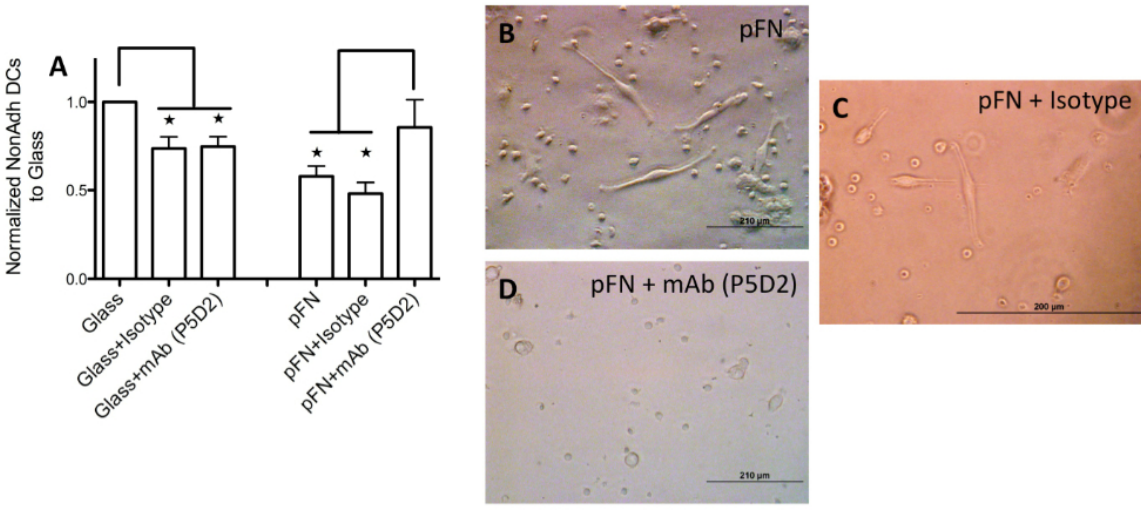

Figure 7-4: Functionality of the purified anti-β_1, the ability to block known β_1-mediated DC adhesion to plasma fibronectin (pFN). Briefly, autoclaved glass cover slips were coated overnight (in 24 well plates) with 10µg/mL human pFN (Sigma) in Hank's balanced salt solution (HBSS) or HBSS only as negative control. DCs at a concentration of 100,000/mL were then pretreated for 1h with anti-β_1 (P5D2) in complete DC media. DCs were then cultured on pFN-coated glass slides for 2h, and non/loosely adherent DC counts were collected and normalized to that collected from glass alone with no pFN-coating (A), mean+s.d. Representative micrographs (n=3) are shown of DCs adhering to pFN in absence of pretreatment (B), pretreated with isotype (C) or anti-β_1 (D) antibody. Stars indicate statistical difference between values and glass alone (1), Student t-test, $p<0.05$. Brackets designate statistical difference between groups, ANOVA $p\leq0.05$ (n=3).

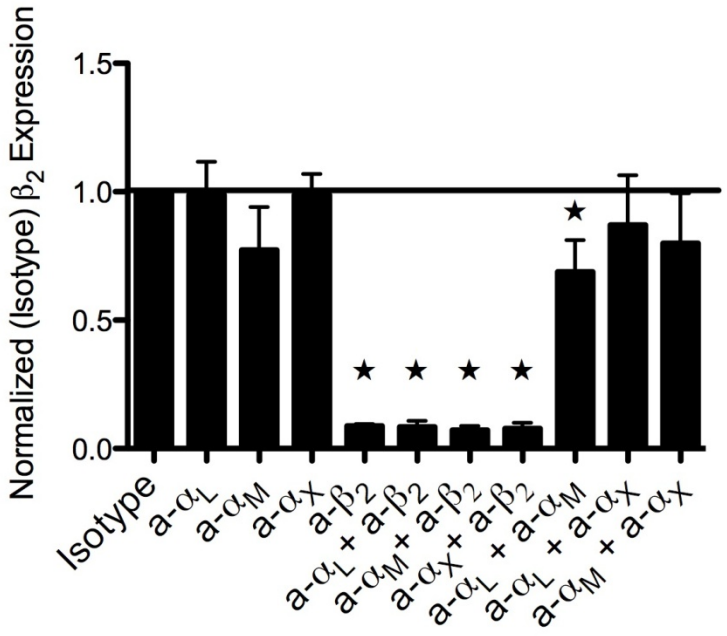

Figure 7-5: β_2 integrin antibody staining on DCs following anti-α and anti-β_2 pre-treatments and treatment with PLGA films for 24h. DCs were stained with anti-β_2-FITC (TS1/18) and expression levels determined via flow cytometry (GMFI) and normalized to that of isotype control. Star indicates significantly lower β_2 staining from that of isotype control, Student t-test, $p \leq 0.05$ (n=3-4).

To investigate whether β_2-mediated DC adhesion plays a role in biomaterial-induced DC maturation, CD86 expression was also examined in β_2-blocked DCs cultured on TCPS or treated with PLGA films. On both substrates, a $CD86^{low}$ population was associated with DCs that were pre-treated with anti-β_2, which exemplifies the appearance of immature DCs (Fig. 7-6A). The percentage of DCs in the $CD86^{low}$ gate (shown in Fig. 7-6A, B) was normalized to that of isotype controls for each donor and averaged for quantification. With increasing concentrations of anti-β_2, DCs became more "immature" as the percentage of $CD86^{low}$ DCs increased for DCs cultured on TCPS or treated with PLGA and particularly at 40μg/mL exhibited significantly higher levels of "immature" DCs on both substrates (Fig. 7-6C).

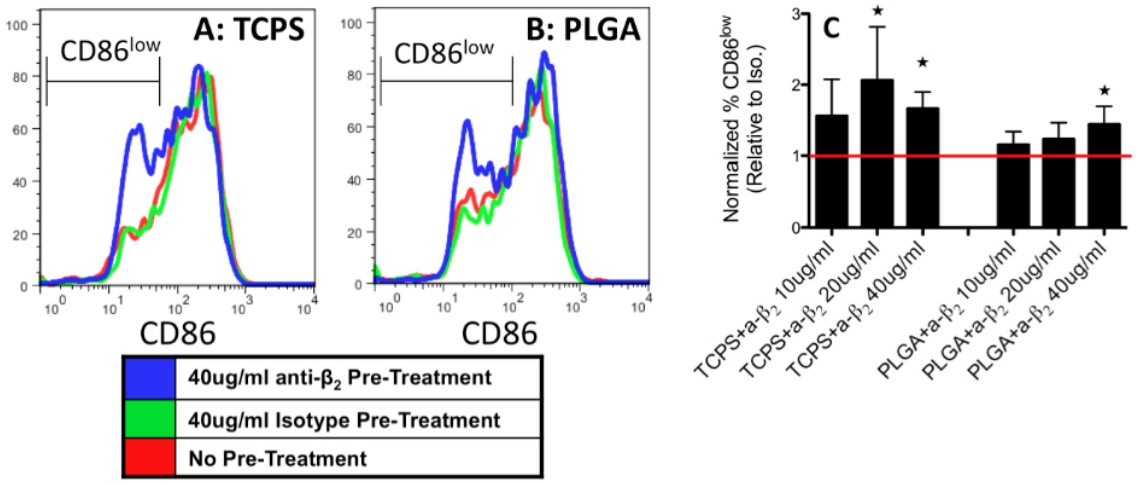

Figure 7-6: DC maturation marker (CD86) expression following anti-β_2 treatment. Representative flow cytometry results of DC CD86 expression at 24h treatment on TCPS (A) or PLGA (B) following pre-treatment of 40μg/mL of anti-β_2 (blue), isotype (green) or no treatment (red). $CD86^{low}$ gate for PLGA was double that of TCPS since PLGA induces increases in CD86 expression. (C) The percentage of DCs in $CD86^{low}$ gate was determined for each donor and normalized to that of isotype controls for each concentration examined. Star denotes statistical difference from isotype (1), Student T-test $p \leq 0.05$ (n=5, mean+s.d).

To attempt to identify the α subunit (α_L, α_M and α_X) which pairs with β_2 and determine the complete integrin molecule responsible for DC adhesion and activation by biomaterials, antibody-blocking techniques were also explored. Blocking antibodies against α_L, α_M and α_X were used along with anti-β_2 and the number of non/loosely-adherent DCs were measured as well as the percentage of $CD86^{low}$ DCs for DCs treated with TCPS or PLGA. For DCs cultured on TCPS, antibodies toward α_L, α_M or α_X alone or in combinations with each other were not able to alter extent of DC adhesion (Fig. 7-7A). Once again, blocking β_2 alone (or in combination with α_L or α_X) significantly increased the number of non/loosely-adherent DCs (therefore, decreasing the number of adherent DCs) (Fig. 7-7A). Surprisingly, anti-α_M treatment seemed to lessen the inhibitory capacity of anti-β_2 to prevent adhesion, which was found to possess similar levels of adhesion to

that of isotype (Fig. 7-7A). As expected, the inhibition of DC adhesion to TCPS by β_2 antibody-blocking matched the reduction in CD86 expression (Fig. 7-7B). Dendritic cells pre-treated with antibodies toward α_L, α_M or α_X showed no shift in the percentage of iDCs ($CD86^{low}$) while DCs pretreated with anti-β_2 (or in combination with α_L or α_X) exhibited a significant increase in the percentage of iDCs (Fig. 7-7B). Also, the anti-α_M pre-treatment, which seemed to prevent anti-β_2 from inhibiting adhesion, similarly appeared to reduce anti-β_2 lowering in CD86 expression by DCs on TCPS to levels found with isotype. For PLGA treatments, similar results were found in that while α subunit antibody-blocking did not affect adhesion (Fig. 7-8A) blocking β_2 alone, or in combination with α_X but not α_L, significantly decreased adhesion and simultaneously lowered CD86 expression (Fig. 7-8A,B). Surprisingly, anti-α_M in combination with either anti-α_L or anti-β_2 showed a slight but significant lowering (compared to isotype) in percentage of $CD86^{low}$ DCs on PLGA which was not found for DCs cultured on TCPS. Overall, while anti-β_2 pre-treatment affected adhesion to a similar degree on both TCPS and PLGA films, it was not as effective in lowering CD86 expression in DCs treated with PLGA as for DC cultured on TCPS (Fig. 7-7B and 7-8B).

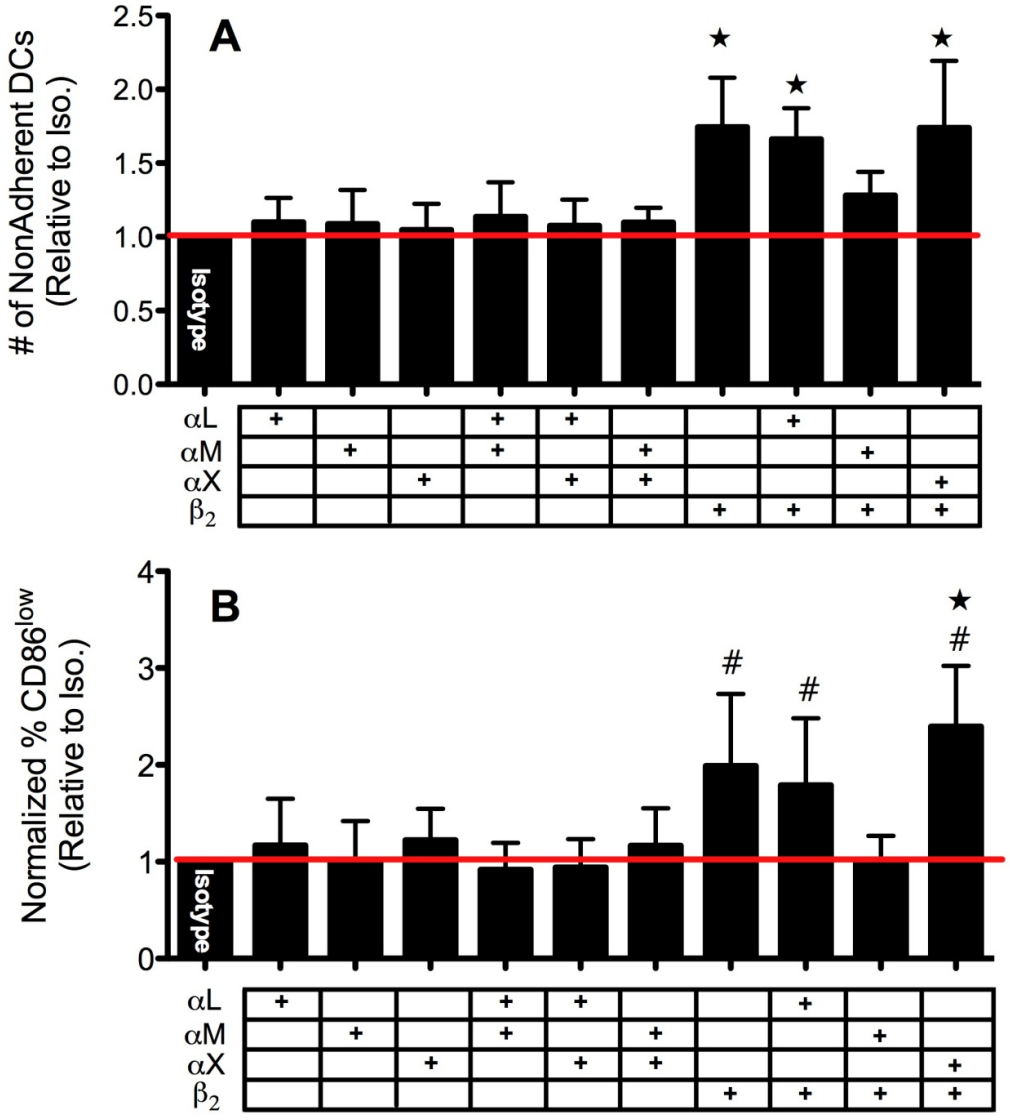

Figure 7-7: DC adhesion (A) and maturation (B) level following anti-$\alpha_L/\alpha_M/\alpha_X$ and/or anti-β_2 blocking on TCPS. (A) Following anti-$\alpha_L/\alpha_M/\alpha_X$ blocking in combination with anti-β_2 (each at 20μg/mL) as denoted by table (+) under graph, non-adherent DC counts were collected. Values were normalized for each donor to that of isotype (1), n=8, mean+s.d. (B) Following anti-$\alpha_L/\alpha_M/\alpha_X$ blocking in combination with anti-β_2 as denoted by table (+) under graph, the percentage of DCs in $CD86^{low}$ gate was determined by flow cytometry and normalized to that of isotype (n=6-8, mean+s.d). Stars for (A) & (B) denote statistical difference from all anti-α combination treatments (ANOVA, $p<0.05$). # designates statistical difference from isotype (1), Student T-test $p\leq0.05$.

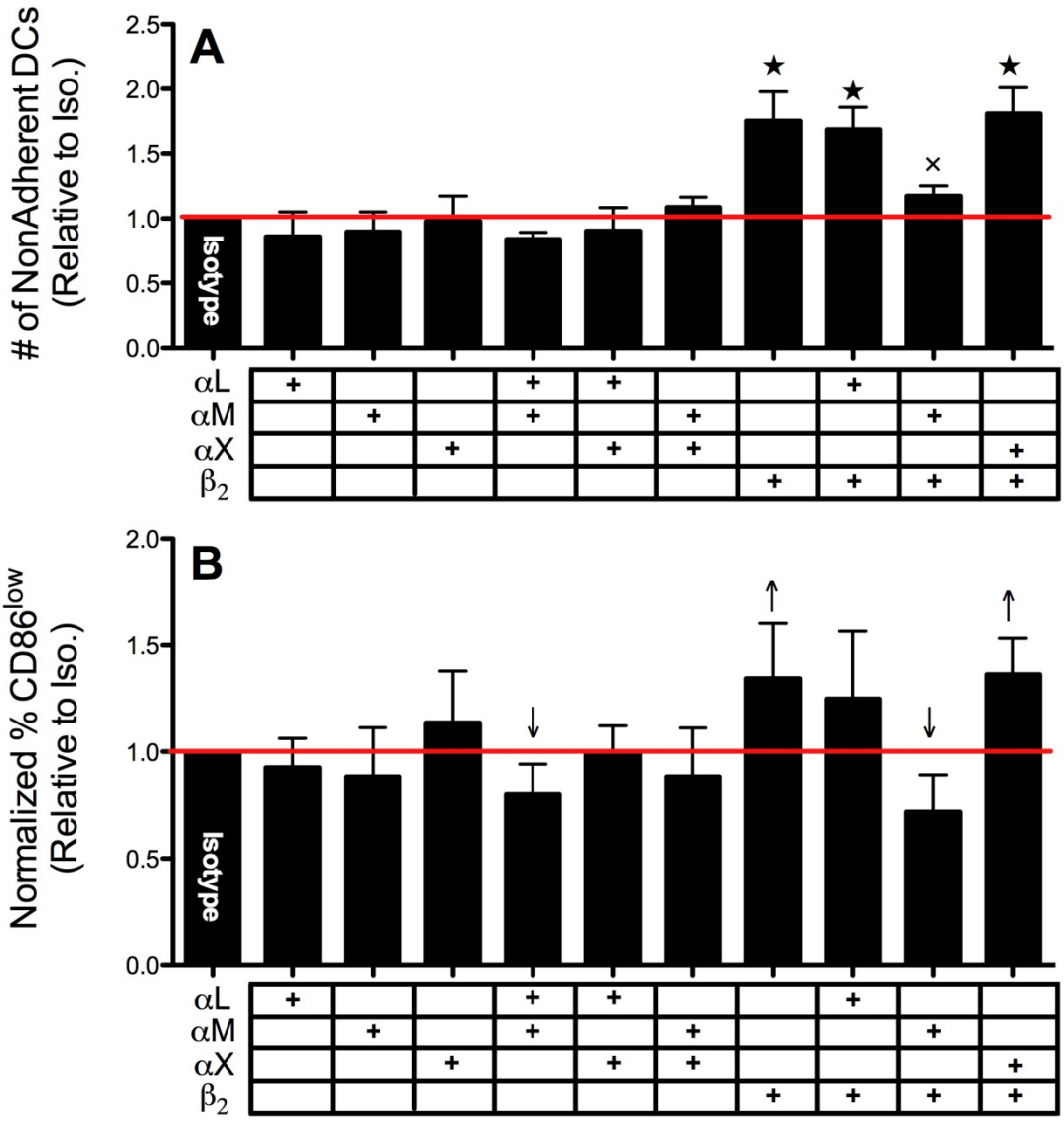

Figure 7-8: DC adhesion (A) and maturation (B) level following anti-$\alpha_L/\alpha_M/\alpha_X$ and/or anti-β_2 blocking data on PLGA. (A) Following anti-$\alpha_L/\alpha_M/\alpha_X$ blocking in combination with anti-β_2 (each at 20μg/mL) as denoted by table (+) under graph, non-adherent DC counts were collected. Values were normalized for each donor to that of isotype (1), n=5-6, mean+s.d. (B) Following anti-$\alpha_L/\alpha_M/\alpha_X$ blocking in combination with anti-β_2 as denoted by table (+) under graph, the percentage of DCs in $CD86^{low}$ gate was determined by flow cytometry and normalized to that of isotype (n=5-6, mean+s.d). Stars for (A) denote statistical difference from all lower values (ANOVA, $p<0.05$). X indicates statistical difference from anti-α_L + anti-α_M (ANOVA, $p<0.05$). Arrows for (B) indicates statistically above or below isotype (1), Student t-test $p\leq0.05$.

Lastly, to further verify the role of integrins in DC adhesion to biomaterials, both β_1 and β_2 integrin expression and their direct interaction with PLGA films was visualized on PLGA-adherent DCs using confocal microscopy. As shown in Figure 7-9, DCs expressed low levels of β_1 integrins which did not appear to co-localize with F-actin (Fig. 7-9A) in DC podosomes. In contrast, co-localization of F-actin with β_2 was commonly present in DCs at the sites of adhesion and spreading on PLGA (Fig. 7-9B). Furthermore, cross-linking adherent DCs to the surface of PLGA and removing non-cross-linked cellular components via an SDS solution showed high-levels of β_2 presence at the cross-linked contacts between DCs and PLGA (Fig. 7-9D). The outlines of DC remnants on PLGA were visible with β_2 visualization but were not observed when examining β_1 (Fig. 7-9C).

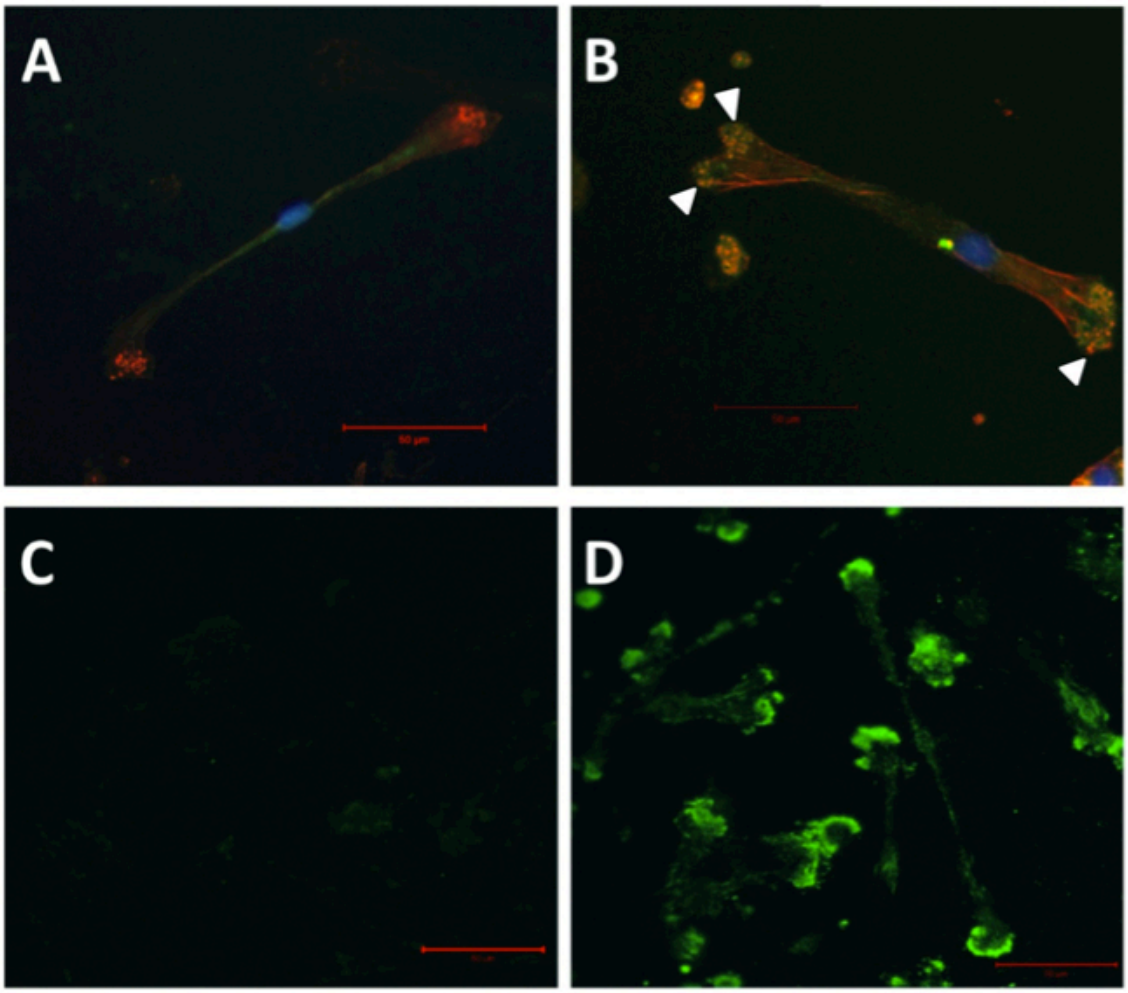

Figure 7-9: β_1 vs. β_2 integrin expression on DCs adhering to PLGA films. DCs were allowed to adhere to PLGA films for 1.5h and subsequent were fixed, permeabilized and stained with either anti-β_1-FITC (A) or anti-β_2-FITC (B) both (green) in combination with phalloidin-TRITC (red) and nuclei (blue). White arrowheads indicate areas of co-localization. To examine receptors at DC-biomaterial interface alone, DCs were treated with PLGA films for 1.5h and cross-linked to surface using DTSSP. Non-cross-linked components were then extracted using 0.1% SDS. Polyclonal antibodies (goat) against β_1 (C) or β_2 (D) were used in combination with anti-goat-FITC secondary to detect integrin presence remaining on films. All films were imaged using confocal microscopy at 40X. Scale bar denotes 50μm.

Discussion

The results presented in CHAPTER 7 illustrate the role and significance of β_2 integrins in the recognition and response of DCs to biomaterials. A biomaterial which supports maturation of DCs, such as PLGA, induced up-regulation of integrin adhesion molecules which is in contrast to the response to surfaces that do not support DC maturation such as agarose or TCPS (Fig. 7-2). This may be related to the increase in DC adhesion which occurred on PLGA in comparison to TCPS substrate (Fig. 7-1). Using antibody-blocking techniques, adhesion to both TCPS and PLGA was found to be β_2-dependent (Fig. 7-3B and 7-3C) and β_1-independent (Fig. 7-3A) which has similarly been shown for monocytes (McNally and Anderson 2002). Notably, a role for β_2 was found not only in adhesion but also in contributing to determining the state of DC maturation as the percentage of $CD86^{low}$ expressing DCs was significantly increased during β_2 antibody blocking of DCs on both TCPS and PLGA substrates (Fig. 7-6, Fig. 7-7, & Fig. 7-8). Biomaterial integrin-mediated adhesion in leukocytes has been established for monocytes/macrophages and neutrophils (Davis 1992; McNally and Anderson 1994; McNally and Anderson 2002; McNally et al. 2007). Particularly, β_2 integrins (but not β_1) are present on monocytes at early time points (1.5h) during adhesion to biomaterials (McNally and Anderson 2002). The current work adds to the field of biomaterials by illustrating the importance of β_2 integrins at not only mediating adhesion of DCs to biomaterials but also more importantly contributing to their maturation state.

Possible α-subunits (α_L, α_M, α_X) were also targeted to determine which may pair with β_2 to mediate such DC response to biomaterials; however, no subunit examined appeared to affect either adhesion or maturation on TCPS (Fig. 7-7) or PLGA (Fig. 7-8). This may be due to anti-α antibodies not binding the appropriate epitope for effective

inhibition of adhesion of DCs to biomaterials. Also, while double combinations of anti-α antibodies were tested (Fig. 7-7 and Fig. 7-8), it is possible that compensatory mechanisms across distinct integrin family members were contributing to adhesion when only one or two α subunits were blocked. A triple cocktail of anti-(α_L, α_M, α_X), which was not tested, may prevent these compensations and potentially decrease adhesion of DCs to biomaterials. However, anti-β_2 treatment in combination with anti-($\alpha_L$or α_X) still lowered extent of adhesion and the state of maturation on TCPS (Fig. 7-7) and on PLGA (Fig. 7-8). Anti-α_M treatment either inhibited or counteracted the effect of anti-β_2 and maintained levels of DC adhesion and maturation to that of isotype controls. One possible explanation is that anti-α_M may preclude anti-β_2 binding through steric hindrance, as noted with flow cytometry for β_2 antibody-staining which is somewhat lowered in the presence of anti-α_M (Fig. 7-5). This seems likely as anti-α_M did not appear to affect DC adhesion or maturation on its own. To assure this, another blocking mAb for α_M (clone CBRM1/5, (Oxvig et al. 1999)) was also examined and was found to not prevent DC adhesion to TCPS while also inhibiting anti-β_2 binding to DCs (data not shown). Though this seems to potentially indirectly link α_M to β_2-mediated DC adhesion to biomaterials, others have shown that MAC-1 ($\alpha_M\beta_2$) requires α_M for spreading while adhesion is primarily mediated through the β_2 subunit (Solovjov et al. 2005). Here, anti-α_M treatment did not lower the extent of DC spreading on biomaterials though it did induce slight clustering (data not shown).

Interestingly, β_1 and β_2 mRNA were both up-regulated in DCs treated with PLGA (Fig. 7-2); however, a role for β_1 was not found while utilizing three known β_1 blocking monoclonal antibodies (P5D2 as well as JB1A, AIIB2). Dendritic cell adhesion to pFN-coated glass, however, was found to be β_1-dependent (Fig. 7-4) as has been previously shown (Swetman Andersen et al. 2006) which suggests that DC adhesion to the

complex serum-adsorbed surfaces on biomaterials may be β_1/FN-independent. The results received through mRNA expression analysis emphasize the need, particularly for integrins, for investigation at the protein level but even further at the cell surface using antibody-blocking or through other techniques such as RNAi. However, it is possible that at times greater than 24h, not studied here, that β_1 protein and surface expression becomes relevant. β_1 integrin expression has been shown to increase and become a relevant factor in adhesion as biomaterial-adherent monocytes differentiate into macrophages at one week (McNally and Anderson 2002). Dendritic cells also have higher levels of expression of β_2 than macrophages (Ammon et al. 2000). These may be important and applicable differences between macrophage and DC interactions with biomaterial surfaces. Biomaterials, designed to limit β_1-mediated adhesion, may allow for DC presence via β_2 but avoid macrophage and foreign-body giant cell accumulation via β_1.

It appears that for DCs, β_2-mediated adhesion is a requirement for biomaterial-induced maturation. Furthermore, even the less-adhesive and less-activating substrate TCPS may require a basal level of β_2 signaling to maintain CD86 expression (Fig. 7-3B). This is an important finding not only for the biomaterials and tissue engineered device community, but also indicates how DCs in general may utilize integrins to maintain their immunostimulatory capacity. It is accepted that DC adhesion and migration ability is heavily influenced by the maturation state. However, these results indicate there is interplay between adhesion and maturation. Others have found that particular adhesive protein substrates, such as collagen (Brand et al. 1998) and many others (Acharya et al. 2008), can induce increases in DC stimulation of T cells. In the current work, the biomaterial component is manipulating the DC phenotype through potentially numerous adhesive ligands presented as part of the adsorbed protein layer on biomaterials.

Surface chemistry of biomaterials is known to influence protein adsorption and conformation to potentially influence differential cellular integrin engagement at the surface (Keselowsky et al. 2003; Keselowsky et al. 2005). Substrate physical properties such as elasticity and hydrophobicity may contribute to both cellular adhesion and subsequent gene expression by altering the adsorption and presentation of adhesive serum ligands for β_2 integrins such as FN or inactive complement component 3 (iC3b) (Wilson et al. 2005; Engler et al. 2006). The PLGA 75:25 material used herein is more hydrophobic than TCPS (71.6^o (Yoshida and Babensee 2006) versus 59^o (Nuryastuti et al. 2009) contact angle, respectively). Others have found that PLGA 50:50, which has an equivalent contact angle to TCPS, 60^o (Lam et al. 2002), does not alone induce DC maturation *in vitro* (Sharp et al. 2009). Thus, hydrophobicity of PLGA 75:25 may explain, in part, why DC adhesion is more prevalent on this composition of PLGA and also potentially why expression levels of CD86 was more difficult to lower for DCs treated with PLGA following β_2 blocking than for DCs cultured on TCPS.

The visualization of integrin presence for DCs upon contact with PLGA films was investigated at an early time point (1.5h) to further verify the role of β_2 integrins in DC adhesion and spreading on a biomaterial surface. β_2 was found in preferentially high levels at podosomes in contact (within 12Å) with biomaterials and co-localized with F-actin (Fig. 7-9B,D). In contrast, the distribution of β_1 was not able to be determined through this cross-linking to PLGA from adherent DCs and was found independent of actin (Fig. 7-9A,C). The morphology and expression patterns are very similar to what has been found on DC podosomes present when cultured on FN-coated surfaces which exhibit β_2, α_M, α_X and actin co-localization while being absent of β_1 (Burns et al. 2004). Here, the authors did not examine the effect of FN-coating on maturation marker expression, but DCs cultured on FN-coated surfaces did appear at least morphologically

similar to DCs treated with PLGA films (Fig. 7-4). While β_1 binding to FN is well known (shown in Fig. 7-4), β_2 integrins, particularly $\alpha_M\beta_2$, have also been established as receptors for FN (Lishko et al. 2003). Thus, the β_2-mediated adhesion to FN may be one possible ligand present upon serum-adsorption to biomaterials, which may play a role in the adhesive response of DCs. Other ligands for β2-integrins include fibrinogen and iC3b; the former is unlikely to be present in the serum used here for culture and active complement activation was prevented by heat inactivation of the serum, but activation fragments could adsorb to the surfaces to mediate recognition.

Lastly, the intracellular link between integrin engagement and DC maturation remains unknown. In monocytes, integrin adhesion to ECM components (such as FN) is known to stimulate nuclear factor (NF)-κB and Jak/STAT signaling pathways (de Fougerolles et al. 2000). The NF-κB pathway plays a particularly important role in inflammatory signaling and, in DCs, controls the expression of numerous DC maturation markers such as CD80 and CD86 (van Vliet et al. 2007). The link between DC integrins and NF-κB is less characterized; however, DCs which were treated with PLGA films for 24h, did not show increases in activated NF-κB presence in nuclear extracts (Yoshida and Babensee 2006). This implies that either NF-κB is not involved in DC adhesion-induced maturation or that NF-κB activation occurs earlier than 24h. However, another explanation is that adhesion alone is not enough to activate DCs, and adhesion simply allows for other co-receptors to be localized to biomaterial surface for engagement. As a an example, toll-like receptor (TLR)-4, a PRR which can recognize not only LPS but many proteins such as fibrinogen (Rock and Kono 2008) and induce DC maturation, is one such potential receptor. In fact, TLR4 has been shown to play a role in leukocyte recognition of biomaterials as TLR4-deficient mice display a delayed biomaterial-adherent leukocyte profile (Rogers and Babensee 2010). Integrin subunit α_M (CD11b)

has recently been found to negatively regulate TLR triggered inflammatory responses (Han et al. 2010); however, the role of other α subunits and β_2 in TLR signaling remain unknown. Here anti-β_2 and isotype pre-treated DCs yielded a similar increase in CD86 expression upon treatment with ultrapure-LPS (data not shown), implying β_2 inhibition may not have influenced TLR4 responsiveness. Overall, while integrin-mediated adhesion may be required for DC maturation in response to biomaterials, other co-receptors may contribute to the state of DCs in contact with biomaterials.

In conclusion, we have shown that DC integrin-mediated adhesion to biomaterials plays a role in determining the maturation state of DCs. β_2-integrins (but not β_1) were involved in DC recognition of both TCPS and PLGA films and blocking β_2 lowered the extent of induced maturation of DCs. This has important relevance in determining criteria for material selection in the field of vaccine design and in the field tissue engineering.

CHAPTER 8

CONCLUSIONS AND FUTURE WORK

Understanding events required for the initial host recognition of biomaterials is critical in design and implementation of effective biomaterial treatments and applications. In particular, elucidation into the mechanism behind the inflammatory response to biomaterials and its role in the adjuvant effect would be of utmost importance to material selection for tissue-engineered devices. Since DCs function *in vivo* to initiate an adjuvant response by linking both an increased innate response to a heightened adaptive immune response, the direct receptor recognition of biomaterials by DCs was investigated. Dendritic cells possess many PRRs, which bind not only PAMPs but also many endogenous molecules ('danger signals' or DAMPs), which are capable of leading to an adjuvant effect by increasing DC maturation. The objective of this thesis research was, in part, to investigate the relevance of biomaterial-associated DAMPs in the overall host response to an implant *in vivo* and the response of DCs to biomaterials *in vitro*. The interactions between host and DC receptors to these endogenous molecules were isolated through the investigation of TLR4. Concurrently, an attempt was made to probe biomaterial surfaces *in vitro* for serum 'danger signal' adsorption. A major finding of this thesis was that TLR4 contributes to the acute inflammatory response to a biomaterial implant *in vivo*. TLR4-deficient mice possess a delayed adherent leukocyte profile on the biomaterial surface in comparison to $TLR4^{+}$ mice. It is believed that biomaterials may act as a depot for DAMPs that are potentially induced following the surgical implantation procedure or produced *in situ* at the biomaterial-interface through adsorption-induced protein denaturation. Biomaterials may, thus, present DAMPs to leukocytes and stimulate acute inflammatory signaling through TLR4; however, other compensatory receptors (potentially other TLRs) may also contribute as seen in the perpetuation of

chronic inflammation (fibrous capsule formation) in TLR4-deficient mice. This finding further adds complexity to the body's response to a biomaterial and how it recognizes a material as a foreign object. The major implication may be that molecules produced by an implantation procedure potentially contribute, at least initially, to the body's response to an implant. Therefore, it is hypothesized that minimizing implant-induced production of DAMPs, through use of minimally invasive surgical techniques, may potentially lessen TLR4's contributions to acute inflammation.

Also, as DC adhesion and integrin expression increased following culture on PLGA but not agarose films *in vitro*, which mimicked the humoral immune response induced by these biomaterials (Matzelle and Babensee 2004; Norton et al. 2010), an investigation into integrin-mediated adhesion in biomaterial-induced DC maturation was employed. Overall, these experiments presented herein aimed to investigate, at the cell receptor level, how the body's innate response toward a biomaterial may influence its adaptive immune response to a co-delivered biologic.

One of the major findings of this thesis work was that β_2 integrin-mediated adhesion to biomaterials contributes to the state of DC maturation. DCs appeared to be utilizing β_2 integrin family members to recognize and respond to biomaterial substrates in a manner that has previously not been identified. This may be an important finding for potentially predicting the adjuvanticity of a biomaterial utilized in a tissue-engineered construct. PLGA, which has been found to elicit an increased immune response to a co-delivered biologic, displayed increased levels of β_2 mediated adhesion by DC *in vitro*. It is believed that increased DC adhesion *in vivo* may contribute to increased maturation and subsequent elicitation of an immune response. Agarose, which does not display similar adjuvanticity to a co-delivered biologic, does not elicit high levels of DC adhesion *in vitro*. Therefore, it is hypothesized that appropriate material selection for a tissue-engineered construct may depend on minimizing the level of DC adhesion in order to

minimize the biomaterial-induced mediation of the immune response. However, in the context of a biomaterial vaccine-delivery vehicle, an increased immune response would be appropriate, and thus, DC recognition and adhesion to a biomaterial would likely be maximized.

Though an overriding biomaterial property contributing to DC adhesion is unknown, it is believed that material properties do influence the adsorption and presentation of plasma/serum proteins to DCs. In the context of this thesis, it is believed that the hydrophobicity of a biomaterial (as stated in CHAPTER 7) may influence the level of DC adhesion to a biomaterial, and thus influence a subsequent supported immune response to an associated biologic. The level of DC adhesion and maturation correlates to increases in contact angle across agarose (0^o), TCPS, (60^o) and PLGA (72^o). A major roadblock for determining the contribution of hydrophobicity to DC maturation across these materials rests in their diverse surface chemistry. The degree of hydrophobicity and its influence on DC adhesion and maturation across one particular material, such as PLGA, may potentially be examined by treating DCs with PLGA with differing lactide to glycolide ratios, which would alter hydrophobicity utilizing similar surface chemistry. Across differing materials, however, no single surface property may be isolated for comparison without modifying their surface chemistry. Therefore, isolating a single surface property of biomaterials, which both influences DC adhesion and helps modulate an immune response, is not likely to be successful.

Another major finding of this thesis was that TLR4 played a role in the acute inflammatory response to a biomaterial implant as seen in the altered adherent leukocyte profile (Fig. 4-4), which seemed to be surface dependent as the milieu surrounding the implant (lavage) displayed similar leukocyte profiles across $TLR4^+$ and $TLR4^-$ mice (Fig. 4-2). It is hypothesized that biomaterial-associated 'danger signal' molecules mediated these effects. Further examination into potential murine

endogenous molecules, which potentiate this response, would be beneficial. PET discs should be pre-adsorbed with known murine DAMP ligands of TLR4 such as HSPs (Facciponte et al. 2005), hyaluronan (Taylor et al. 2004), denatured fibronectin (Okamura et al. 2001), or fibrinogen (Smiley et al. 2001), and the differential adherent leukocyte profiles determined following 16hr implantation into $TLR4^{+}$ and $TLR4^{-}$ mice. This technique has been used to determine the role of fibrinogen in the acute inflammatory response to biomaterials (Tang and Eaton 1993). Molecules which induce heightened acute inflammatory response, as seen in both increased leukocyte adhesion and increased monocyte/macrophage presence, may point towards relevant 'danger signals' interacting with TLR4. In contrast, a specific molecule's role in the host response to biomaterials may be investigated using knockout-mice for molecules such as HSPs [e.g. $hsp72^{-/-}$ mice (Oh et al. 2004) which is known to interact with TLR4 (Williams and Ireland 2008)], using biomaterial implantation techniques as investigated herein.

As other TLRs besides TLR4, such as TLR2, are capable of recognizing endogenous 'danger signals' (Rock and Kono 2008), the role of many TLRs in the host response to a biomaterial implant should be investigated using $MyD88^{-/-}$ mice, which have impaired TLR responsive due to the lack of the common adaptor molecule (Kaisho and Akira 2001). These mice display severely inhibited DAMP-mediated inflammation (neutrophil recruitment) in the peritoneal cavity following injection of necrotic cells (Chen et al. 2007). It is hypothesized that mice lacking MyD88 would show severely inhibited acute inflammatory following biomaterial implantation through lack of TLR-induced inflammation. Furthermore, unlike with TLR4-deficient mice used here, which displayed persistent chronic inflammation similar to that of wild-type mice through potential compensatory mechanisms by other TLRs, $MyD88^{-/-}$ mice would also inhibit other TLR/ligand signaling. Thus, $MyD88^{-/-}$ mice are anticipated to display inhibited fibrous capsule formation, unlike in TLR4-deficient mice (Fig. 4-6 and Fig. 4-7). Due to identical

signaling pathway shared between TLRs and IL-1R family members, however, the discrepancy between these two receptor families would need further investigation. This should be done with simultaneous examination of the host response to biomaterials in IL-1R$^{-/-}$ mice (Labow et al. 1997) to assure TLR-specificity.

Direct TLR4 interaction with biomaterial surfaces *in vivo* following 16hr intraperitoneal implantation should further be determined to assess if TLR4-biomaterial interactions are potentially the source for controlling adhesive leukocyte profile. This should be done using similar techniques as described in CHAPTER 7 for cross-linking integrins to the biomaterial surface (Keselowsky and Garcia 2005). Briefly, PET discs implanted in the peritoneal cavity in TLR4^{+} mice for 16hr would be explanted and rinsed three times in D-PBS to remove non/loosely adherent cells. Subsequently, discs would be submerged in a solution of 0.1% SDS for 5m to gently extract cellular material. Remaining bound receptors on PET discs would be cross-linked to the surface using 1mM DTSSP (12Å, Pierce). To assess the presence TLR4, a polyclonal antibody toward murine TLR4 (Abcam) would be added to biomaterial surface for 1hr followed by a fluorophore conjugated secondary antibody. Biomaterial surface would be mounted and presence of TLR4 assessed via confocal microscopy via comparison to non-crosslinked control surfaces. This should also be done for similarly implanted PET discs in TLR4-deficient mice to assure the specificity of the primary antibody. As DCs were shown to utilize β_2 integrins to recognize and adhere to the biomaterials *in vitro* (Fig. 7-6C, Fig. 7-7, and Fig. 7-8), these integrins could be verified *in vivo* using the same cross-linking technique to illustrate direct integrin engagement by adherent cellular material (though not necessarily DC-specific).

An attempt was made to determine the differential presence of biomaterial-adsorbed serum proteins across PLGA and agarose surfaces, which may mediate their distinct DC responses *in vitro* (APPENDIX 3). However, while it was found that protein

could be eluted from the biomaterial surfaces, equivalent loading (as determined via protein assay) across individual eluates run on western blots could not be assured (Fig. A3-2). So while eluates from agarose surfaces were found to possess higher presence of IgG (Fig A3-3), this is most likely due to the increased loading of total protein. In future work, to determine the presence of adsorbed serum proteins, such as fibronectin, across different biomaterials, radiolabeled-versions (I^{125}) of these molecules should be spiked into 10% serum prior to adsorption on PLGA or agarose films. Following three vigorous PBS rinses of films, presence of radiolabeled proteins would be determined using a scintillation counter. It is anticipated that PLGA, a DC activating material, would induce increased adsorption of many serum proteins in comparison to agarose. Also, an ELISA-based detection method, previously used to detect the presence of adsorbed fibronectin, may also prove useful (Grinnell and Feld 1982).

Though the role of DCs in the host response to biomaterials is presumed, further assessment of their involvement in initial biomaterial recognition should be investigated. Currently, the Babensee lab is examining the role of DCs in the biomaterial adjuvant effect using genetically engineered mice possessing CD11c-dependent diptheria toxin (DT) receptor expression (Jung et al. 2002). By delivering DT, mouse DCs ($CD11c^+$) may be temporarily depleted, and their role may be assessed during both the innate and adaptive immune response to a combination product. Though DC ($DEC\text{-}205^+$) presence is found in the cellular infiltrate of implanted PLGA scaffolds in mice (Babensee 2008), direct DC recognition of biomaterials *in vivo* has yet to be examined as has been shown *in vitro* (CHAPTER 7). This should be done utilizing implantation of PLGA discs (1cm) in the peritoneal cavity of C57BL/6 mice and examination of bound cellular fraction at early time points (1-24hr). Following explant and three separate D-PBS rinses, adherent cellular fraction should be fixed using 4% paraformaldehyde (Polysciences Inc). Adherent cells on PLGA should be permeabilized with Triton X-100 as described in

CHAPTER 7 and stained using fluorophore conjugated antibodies for DC markers DEC-205 and CD11c as well as phalloidin for cytoskeleton determination and DAPI nuclei stain. Using confocal microscopy, one could determine the presence of DCs (up to 24hr), which are directly recognizing the biomaterial *in vivo*. This would further contribute to elucidating the role of DCs in the inflammatory response to biomaterials.

In the context of DC-biomaterial interactions *in vivo*, while the direct recognition may determined through microscopy, the response (or maturation) of DCs to biomaterial implantations should also be investigated. As distinct biomaterials, such as PLGA or agarose, may differentially influence an immune response (Bennewitz and Babensee 2005; Norton et al. 2010), DC presence and response to both PLGA and agarose should further be examined *in vivo*. This should be examined following a 16-24hr implantation of either PLGA or agarose discs (separately) in the peritoneal cavity of C57BL/6 mice. Though biomaterial-induced DC maturation may require adhesion *in vitro* (CHAPTER 7), even non/loosely adherent human monocyte-derived DC fraction still exhibit signs of maturation potentially through paracrine effects initiated by the adherent fraction (Yoshida and Babensee 2004). In an *in vivo* intraperitoneal implantation setting, this relates to biomaterial-adherent cells eliciting a response to cells in the surrounding milieu, which may not be in direct in contact with the biomaterial. Therefore, both biomaterial-adherent and lavage DC presence and response to PLGA or agarose disc implantation should be investigated.

Prior to explanation, peritoneal cells should be collected via PBS lavage, as performed in CHAPTER 4. Peritoneal cells should be examined for DC presence via flow cytometry through CD11c or DEC-205 expression in comparison to isotype controls. Following the determination of the DC population from both DC marker expression and size from forward scatter via flow cytometry, DC maturation marker expression should concurrently be determined by double staining with anti-CD86. While the analysis of

lavage cells for DC presence and response by flow cytometry may be trivial, biomaterial-adherent cell fractions may be difficult to collect for similar analysis. First, trypsin addition followed by light cell scraping to explanted PLGA and agarose discs to collect adherent cells should be attempted as described in CHAPTER 4. Biomaterial-adherent cell fractions should subsequently be examined for CD11c and CD86 expression via flow cytometry. Relative levels *in vivo* of biomaterial-induced DC maturation, determined via CD86 expression, should be compared between PLGA and agarose implants. Also, comparison to sham surgery and naïve control mice should be used to assess biomaterial-induced responses specifically. It is speculated, however, that cell collection using trypsin may destroy the extracellular domains of both CD11c and CD86. If this occurs, then collection of biomaterial-adherent cell fractions may require a non-enzymatic cell dissociation solution (Sigma) with vigorous pipetting to assure collection of cells for flow cytometric analysis.

Human monocyte-derived DCs and murine BMDCs interestingly displayed very different adhesion-mediated maturation responses. Human monocyte-derived DCs seemed to require β_2-mediated adhesion to biomaterials in order propagate biomaterial-induced maturation, and in fact inhibiting adhesion to surfaces resulted in lower DC maturation marker expression (CHAPTER 7). In contrast, adherent BMDCs showed increased signs of immaturity and had significantly increased responsiveness to both LPS and biomaterial stimulation in comparison to non/loosely adherent BMDCs (CHAPTER 6). The reason for this discrepancy between human and murine derived DC adhesion and responsiveness to biomaterial stimuli is unknown, but future work may help determine its nature.

One possible explanation is that BMDCs adhere to biomaterial surfaces using different integrins than human monocyte-derived DCs, which may allow for maintenance of their immature status following adhesion. CD11b(α_M)-deficient macrophages are more

responsive to maturation-inducing stimuli (TLR ligands) due to a negative cross-talk found between α_M engagement and TLR signaling pathway (Han et al. 2010). CD11b(α_M)-mediated adhesion to surfaces in BMDCs would thus decrease their responsiveness to ultrapure-LPS. The opposite was found for adherent BMDCs (Fig. 6-5, and Fig. 6-6), and therefore, it is unanticipated that α_M is involved in the adhesion to biomaterial surfaces. In fact, BMDC adhesion to an increasing surface density of RGD-peptides led to increased signs of maturation and slightly correlated with α_V integrin binding (Acharya et al. 2010). For human monocyte-derived DC adhesion, it was shown that β_2 integrins mediate adhesion contribute to biomaterial-induced DC maturation (Fig. 7-6, Fig. 7-7, and Fig. 7-8); however, an integrin α subunit partner was not definitively discovered. Anti-α_M antibody treatment did significantly prevent anti-β_2 inhibition of adhesion and reduction in maturation (Fig. 7-7 and Fig. 7-8) through potential steric hindrance of anti-β_2 binding, as supporting evidence hinted (Fig. 7-5). It is possible that this slight steric hindrance implies a role for α_M (and thus MAC-1, α_M/β_2) in human DC biomaterial interactions but it was not able to determined using antibody-blocking techniques.

To screen different integrins utilized by human monocyte-derived DCs and BMDCs to attach to biomaterial-surfaces, both DC types should be cultured on PLGA films and cross-linked using DTSSP following SDS extraction as described previously (CHAPTER 7). Direct binding between integrin α subunits and biomaterial surfaces should be identified using fluorophore conjugated polyclonal antibodies against α_M, α_L, α_X and α_V as well as β_2 and β_1 integrins and compared to non-cross-linked controls. This could be a starting point for determining relevant integrins involved in differential interaction between human monocyte-derived DCs and BMDCs. This technique would also be a powerful tool to analyze surface binding of potential DC maturation-inducing

receptors (such as Fc-receptors or CLRs), which may be involved in the direct recognition of biomaterials and used as a preliminary investigation into their relevance of DC/biomaterial interactions. However, the cross-linking reagent used may have to be altered (lengthened or shortened from length of DTSSP, 12Å) to assure proper connection with the appropriate receptor of interest.

Lastly, though β_2 integrin-mediated adhesion was shown to influence the maturation state of DCs (CHAPTER 7), a direct link between adhesion and maturation was not determined. Further analysis would be required to attempt to decouple integrin-inducing signaling from DC maturation. To accomplish this, a technique employed by Miyamoto et al utilizing non-inhibitory monoclonal integrin antibodies has been shown to induce integrin clustering and adhesion without the induction of major integrin intracellular signaling (Miyamoto et al. 1995; Miyamoto et al. 1996). This may be tested in DCs by coating substrates (such as TCPS) with distinct monoclonal antibodies against integrin subunits (both α and β_2 subunits), which should allow for integrin-mediated adhesion, but not allow intracellular recruitment of signaling components such as talin. DC adhesion to the coated substrate would assessed using cell counts as well as confocal microscopy which would also allow for examination of key phosphorylation sites of intracellular molecules (e.g. FAK) to assure integrin signaling was not propagated. The time point of analysis is critical as phosphorylation of intracellular molecules may occur in as little as 5-15 min following treatment. Also, Fab fragments of antibodies may be required for coating to avoid Fc-receptor interaction and stimulation of DCs. Following assurance of integrin binding and adhesion without subsequent signaling, the DC maturation state following treatment on antibody-coated surfaces would be assessed for maturation marker expression (CD80/CD86), at potentially a later time point (>1hr) to determine specifically if DC integrin-mediated adhesion is alone capable of inducing maturation.

If integrin-adhesion alone is not responsible for the maturation of DCs on biomaterials, a major roadblock may exist in attempting to decouple collaborative receptors, which may be involved in the response of DCs to biomaterials. There are numerous maturation-inducing receptors, which may be involved in the recognition of biomaterial-associated molecules as noted in Section 3.5. The research presented here focused on the role of integrins and TLRs because of their major role in adhesion and maturation of DCs, respectively. However, other receptors (e.g. Fc-receptors, scavenger receptors, CRLs) may contribute to the maturation response of DCs to biomaterials. While individual members of these receptor families may be assessed through the use of antibody-blocking techniques, which is not feasible owing to the number of potential receptors, pinpointing an entire family of receptors through a screening process would be critical in order narrow the scope of the research. Families of receptors could be screened by inhibiting signaling molecules, which are common across a particular family, such as MyD88 for TLRs or FAK for integrins. Inhibition would likely be accomplished via RNAi techniques. However, a roadblock for this analysis in DCs is that the technique required for inhibition may alone influence the maturation state of DCs. For example, current work in the Babensee lab has shown that electroporation of human monocyte-derived DCs, which is required for the delivery of siRNA in primary DCs, may induce both maturation/necrosis of DCs. Though certain aspects of electroporation may be fine-tuned to minimize these detrimental effects, it is possible that successful delivery of siRNA, which would be required to specifically inhibit families of receptors in a screening process, may not be compatible with maintaining an immature DC phenotype. Therefore, a major roadblock may exist which will not allow for screening of families of receptors that may collaborate with integrins for DC recognition and response to biomaterials.

The research presented in this thesis aims to address how the body's initial inflammatory response to a biomaterial may influence its subsequent immune response

in the context of a combination product. For this endeavor, DC receptor recognition and response to biomaterials was investigated to elucidate the mechanism behind the biomaterial adjuvant effect. The sum of these results allows for further understanding of biomaterial-induced host responses.

APPENDIX 1

A.1 HOST RESPONSE TO BIOMATERIAL IMPLANTATION IN C57BL/6 MICE

PET discs (10mm) were implanted into C57BL/6 mice (The Jackson Laboratory) as described in CHAPTER 4 for 16hr. IP lavage was performed and total and differential leukocytes counts were determined (Fig. A1-1A). Also, the biomaterial-adherent leukocyte population was similarly assessed for total and differential leukocyte profile (Fig. A1-1B). Both the IP lavage and adherent leukocyte profiles were consistent with $TLR4^-$ control strain C57BL/10 at 16h. IP lavage consisted primarily of neutrophils (Fig. A1-1A) while biomaterial-adherent leukocyte population was made up of equivalent fractions of monocyte/macrophages and neutrophils (Fig. A1-1B). This is in contrast to the $TLR4^-$ strain (C57BL/10ScN) which showed significantly higher fraction of neutrophils and significantly lower fraction of monocyte/macrophages at 16h in adherent leukocyte profile in comparison to $TLR4^+$ C57BL/10 mice. This may further indicate a delayed inflammatory response to biomaterials in $TLR4^-$ mice as noted in CHAPTER 4.

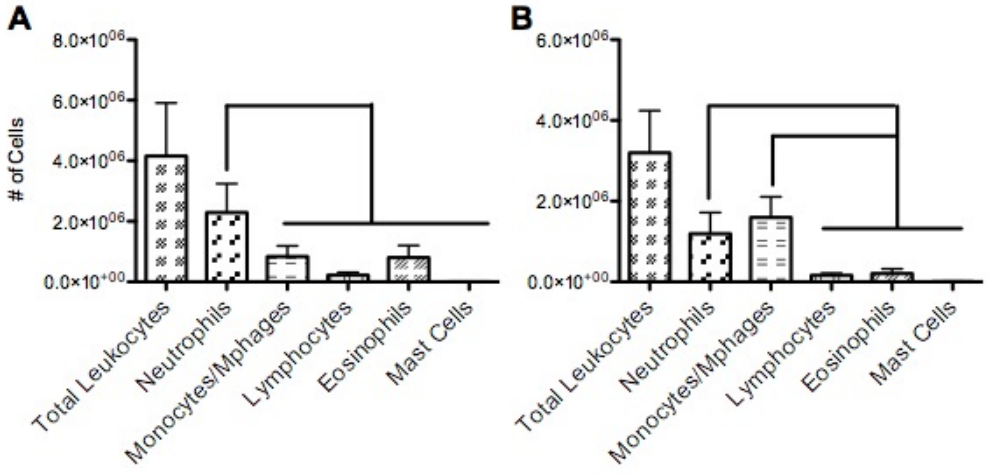

Figure A1-1: Total and differential leukocyte profiles in C57BL/6 mice. These were determined in IP lavage (A) or adhering to biomaterial (B) following a 16hr IP implantation of a PET disc. For detailed description of method, see CHAPTER 4. Brackets indicate $p<0.05$, ANOVA. Bars indicate mean+s.d. (n=6).

APPENDIX 2

A.2 PURIFICATION OF HUMAN DCS USED FOR GENE EXPRESSION OR LYSATE PREPARATION

In order to determine DC-specific gene expression either at the RNA or protein level, pooled adherent and non/loosely human DCs were purified following biomaterial or LPS treatment for 24hr. To do this, magnetic beads (MACS) isolation was utilized which positively selects DC-SIGN (a human DC marker) expressing cells. For the detailed procedure, please see CHAPTER 5. Following isolation of DCs using MACs, purity was assessed via flow cytometry through expression of DC-SIGN and CD19. The primary contaminating cell population in human monocyte-derived DC culture is B cells. Therefore, pre- and post-isolation levels of DC-SIGN and CD19 (B cell marker) were determined (Fig. A4-1). Routinely, >95% of cell population post-isolation was DC-SIGN^{+} while only about 1% of population was CD19^{+}. Pre-isolation percentages of relative populations of DCs and B cells were donor dependent; however, the purification procedure maintained similar percentages of DCs across all donors after purification. These purification results were consistent for isolation of DC used for both RNA extraction and protein level determination from whole-cell DC lysates.

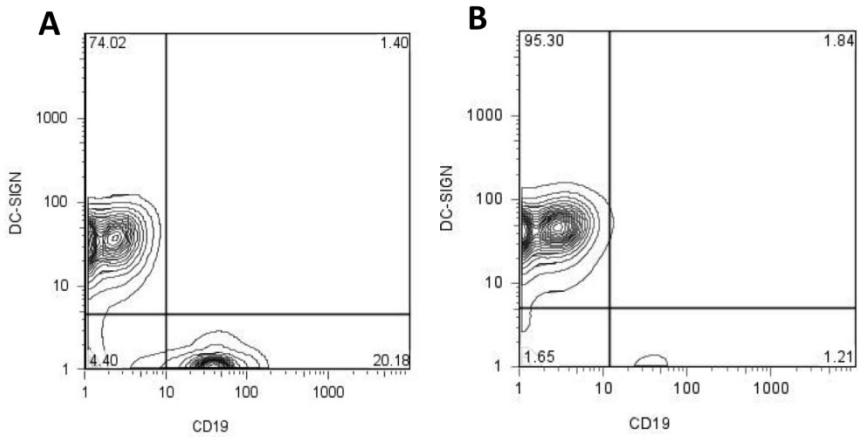

Figure A2-1: Purification of DCs using DC-SIGN microbead isolation. Pre-isolation (A) and post-isolation (B) levels of DC-SIGN vs. CD19 cellular population. Cells were double-stained with DC-SIGN-FITC (R&D Systems) and CD19-APC (Miltenyi Biotec) antibodies were stained. Following isolation, cell population was routinely >95% DC-SIGN^{+} and generally about 1% CD19^{+}. This purification procedure was used for both RNA extraction and DC whole-cell lysate preparation.

APPENDIX 3

A.3 ELUTION OF SERUM PROTEINS FROM BIOMATERIALS

In Specific Aim 1, it was hypothesized that serum adsorbed TLR4/ 'danger signal' interactions may mediate the response of DCs to biomaterials. Numerous plasma/serum proteins such as FN (Okamura et al. 2001; Lasarte et al. 2007), fibrinogen (Smiley et al. 2001), and heat shock proteins (Asea et al. 2002; Tsan and Gao 2004) are known to stimulate cells (specifically DCs) through TLR4, and this may offer an explanation as to why certain biomaterials, such as PLGA, trigger DC maturation while other materials, such as agarose, preserve DCs in an immature state. Therefore, an attempt was made to elute adsorbed serum proteins from the surface of biomaterials in order to determine if specific 'danger signal' proteins were present in higher levels on biomaterials which are known to induce DC maturation.

10% (w/v) PLGA films or 3% (w/v) agarose films were prepared 6 well plates (Corning, Costar) as described in CHAPTER 5. A 10% (v/v) human serum solution in PBS (Invitrogen, pH 7.2) pooled from 3 donors and heat-inactivated (56^{o}C, 30 min) was prepared. Serum solution was stored at -20^{o}C prior to making this solution. Biomaterials, in 6 well plates, were incubated with the 10% serum solution (3mL) for 1hr at room temperature. Uncoated TCPS controls were also incubated with serum solution. Serum was removed serum via pipette aspiration and collected. PLGA and agarose films were carefully removed with clean forceps and transferred to another 6 well plate for washing. The films (and TCPS of original plate) were rinsed three times (5 min each) with PBS (Sigma) (1mL for each wash) at room temperature flipping the films over carefully each time. The films were then transferred to a fresh 6 well plate and surfaces (films and original TCPS well) were treated with 10% SDS/2.3% dithioerythritol (Sigma) mixture on a shaker (1mL per well) for 24hr at room temperature. Samples of pre-incubated serum,

serum removed from surfaces (eluates) and non-adsorbed protein fraction (removed during wash) were saved and stored in 1.7ml low-retention tubes at -20^{o}C.

In order to determine protein concentration of eluates (which has high SDS and DTE presence), a protein assay (Bradford-based) was developed accordingly which would accurately determine levels of protein in serum alone or protein-containing eluates. Standards of BSA (Bio-Rad) from 1.5μg/μL to 0.2 μg/μL were prepared in the SDS/DTE solution (same as eluates). 100μL of standard or samples were placed in acetone-compatible microcentrifuge tube (polypropylene). 400ul of ice-cold acetone were added to each, vortexed and incubated at -20^{o}C for 30 min. These were then centrifuged for 10 min at 15,000g. Tubes were then turned upside down on soak pad to drain and dried for at least 10min until acetone had evaporated. The protein pellet was resuspended in 100μL of water. 40μL of this resulting solution was placed into a fresh tube and 1mL of Bradford reagent (Sigma) was added to each tube. These were incubated for a several minutes and then transferred (200μL) to a 96 well plate for optical density determination at 595nm.

Three independent trials were completed and eluates collected following serum adsorption to TCPS, PLGA or agarose surfaces. Using the protein assay described above, protein concentrations were able to be accurately determined within the range of the assay (1.5μg/μL to 0.2μg/μL) for all eluates. As shown in Fig. A3-1, eluates contained very low levels of protein (between 0.2-0.5μg/μL) depending on the surface; however, no statistical difference was found across the surfaces. This work was completed with the assistance of an undergraduate student Elizabeth Bosworth.

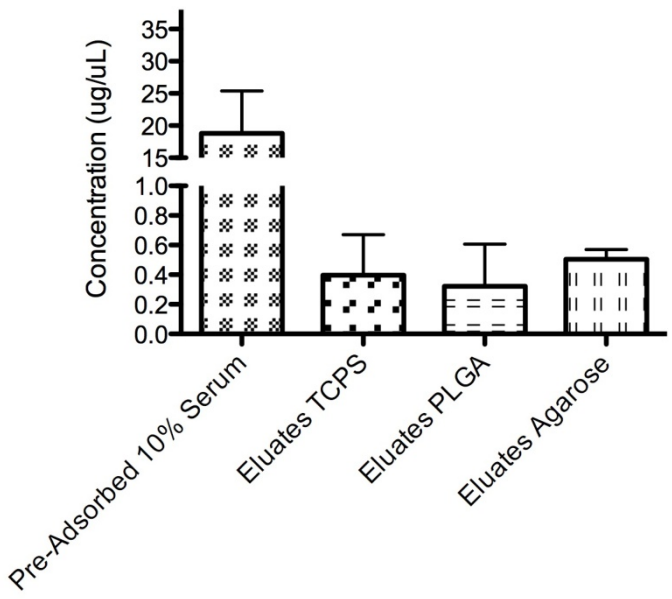

Figure A3-1: Protein concentration determined for pre-adsorbed 10% serum (pooled from three donors), as well as eluates from TCPS, PLGA or agarose surfaces. 10% serum samples were diluted to a range that was within the standard curve to accurately determine the concentration while eluates were run undiluted. Bars are mean+s.d., n=3.

To assure equivalent protein loading in gels across all samples, 3µg of protein from each sample was loaded into SDS-PAGE gels and transferred to a nitrocellulose membrane, as described in CHAPTER 5. The membrane was removed from transfer apparatus and immediately stained with Colloidal Gold Total Protein Stain (Bio-Rad) for 1hr. Membranes were quickly washed and imaged. As shown in Fig. A3-2, though the eluates and 10% serum samples were "equivalently" loaded, there is a significantly higher protein presence in eluates from agarose surfaces than in all other lanes. This was also found when staining similarly loaded gels with Coommassie blue (data not shown). Initial western blots to determine protein presence of IgG in eluates from TCPS, PLGA or agarose showed only significant IgG presence for agarose eluates (Fig. A3-3). The higher protein level in the agarose eluates could explain this result. The reason for

the higher protein presence in eluates agarose surfaces run through gel may be that agarose by-products from the elution procedure interfere with protein assay and cause under-estimation of the protein concentration. However, this was briefly examined and found that eluates from agarose surfaces with no serum adsorption added to proteins of known concentration did not significantly affect the protein assay (data not shown). Therefore, the reason for agarose-eluates showing higher levels of protein than TCPS or PLGA remains unknown. Without reliance of equivalent protein loading during gel electrophoresis, the outcome of any immunoblotting for specific biomaterial-adsorbed proteins (Fig A3-3) cannot be assured. For this reason, further investigation was not undertaken.

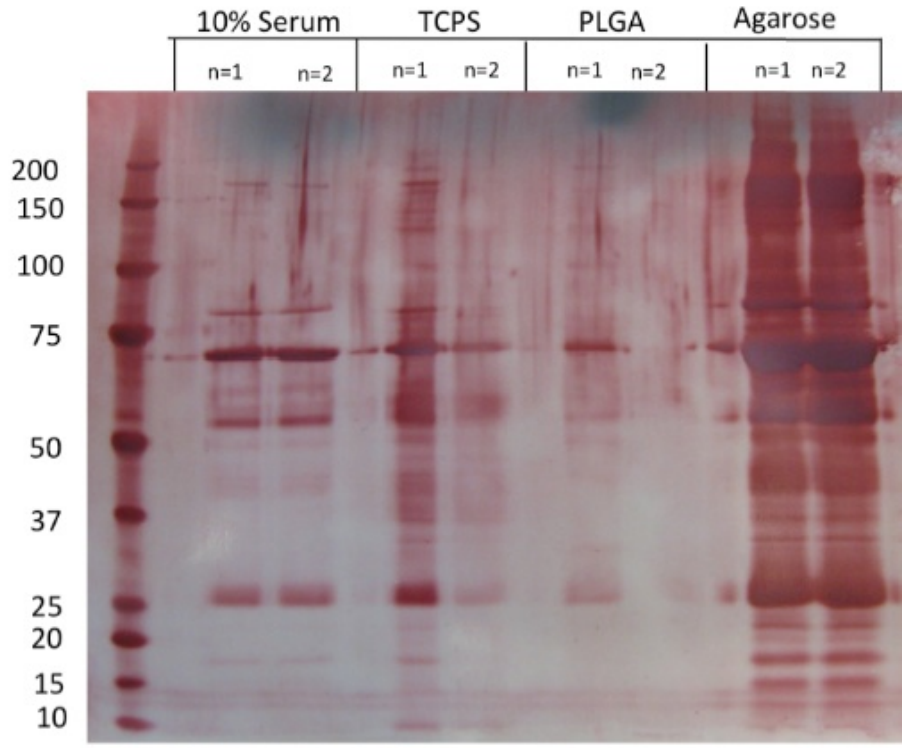

Figure A3-2: Gold stained nitrocellulose membrane of 10% serum, or eluates from TCPS, PLGA or agarose surfaces. 10-200kDa standard was also simultaneously run (left). Note the much larger protein presence in eluates from agarose surfaces compared to TCPS or PLGA.

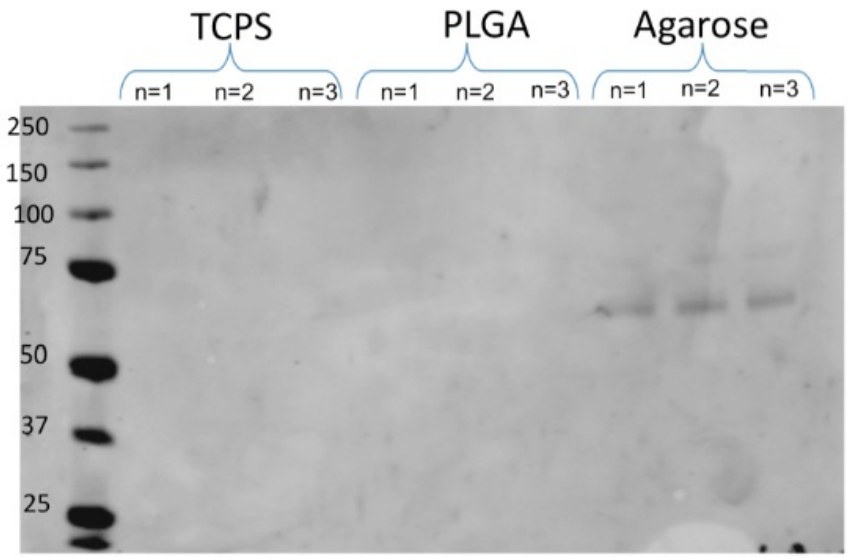

Figure A3-3: Western blot for IgG determination of eluates from TCPS, PLGA or agarose surfaces from three independent trials. 10-200kDa standard was also simultaneously run. Note the much larger IgG presence in eluates from agarose surfaces compared to TCPS or PLGA.

APPENDIX 4

A.4 CHEMOKINE RECEPTOR EXPRESSION HIERACHERCAL CLUSTER ANALYSIS

Chemokine receptors (CCRs) are important markers for *in vivo* phenotype and play an important role in cellular honing (Allen et al. 2007). For this, CCR expression analysis may be able to further yield descriptive *in vivo* significance for where DCs hone or how they react following interactions with biomaterials. CCR RNA expression levels from purified human DCs treated with LPS, PLGA or agarose films were measured simultaneously with adhesion and integrin related molecules using the same RT-PCR techniques as described in detail in CHAPTER 7. Primers used for PCR were also chosen from Harvard PrimerBank (Wang and Seed 2003) and are listed in Table A4-1.

Table A4-1: Primer pairs for chemokine receptors and house keeping genes. Primers were chosen from Harvard Primer Bank (Wang and Seed 2003) with PrimerBank ID listed in table. For detailed information/sequences on each primer pair, please see http://pga.mgh.harvard.edu/primerbank/.

Gene	PrimerBank ID
CCR1	4502631a1
CCR5	4502639a1
CCR6	14043040a1
CCR7	4502641a1
CXCR1	4504681a1
CXCR2	4504683a1
CXCR4	3059120a1
CXCR5	4502415a1
Beta Actin	4501885a1
B2M	4757826a1

Following $-\Delta C_t$ normalization, as described in CHAPTER 7 to β-actin and B2M, a 2D hierarchical cluster analysis was performed using Matlab (v. 2009a, MathWorks) as shown in Fig. A4-1.

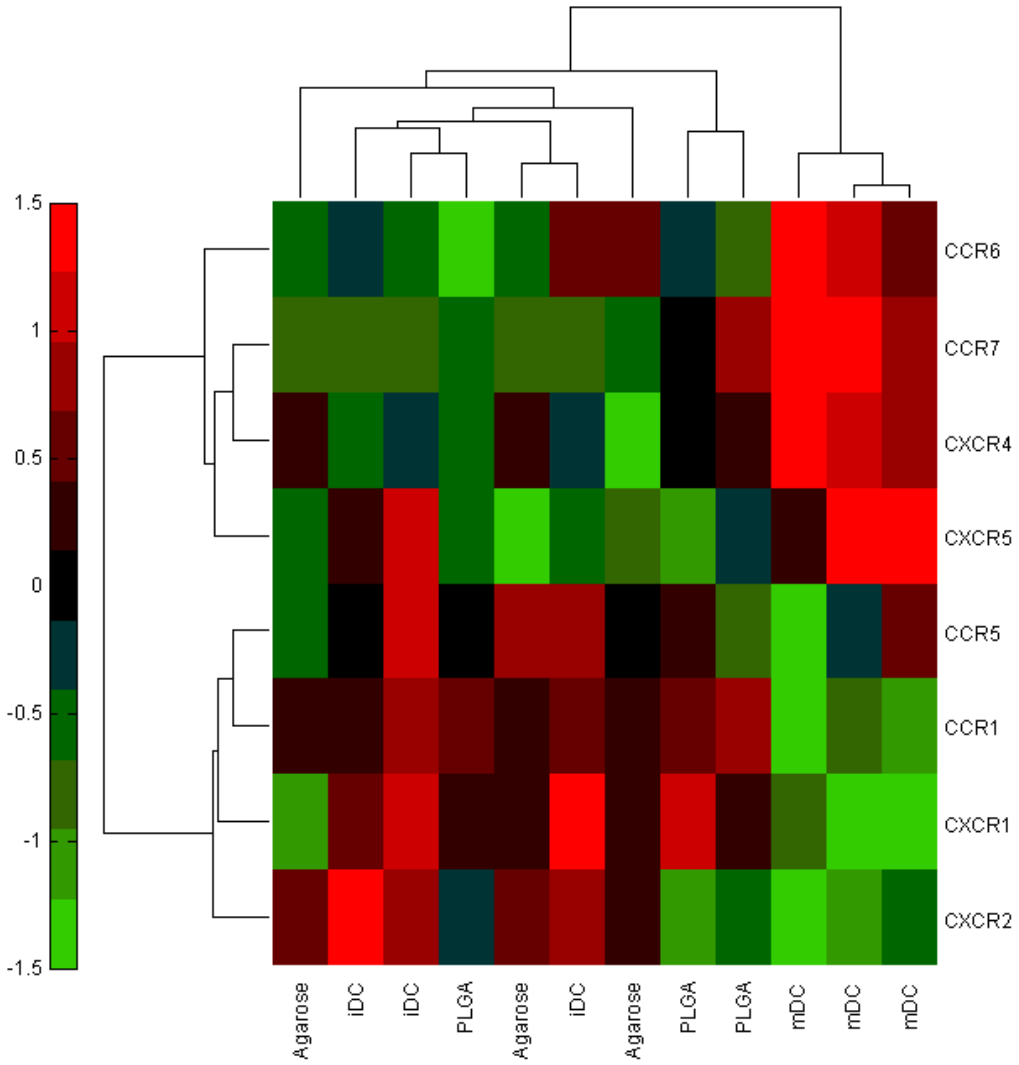

Figure A4-1: Hierarchical 2D cluster analysis for CCR expression. These were investigated simultaneously with integrins and adhesion-related signaling molecules as described in CHAPTER 7. β-actin and B2M C_t values were used for normalized and $-\Delta C_t$ for each trial and treatment were run through *clustergram* analysis via Matlab.

iDC and agarose treatment resulted in very similar CCR gene expression patterns as appeared for both TLR (CHAPTER 5) and integrins (CHAPTER 7) (Fig. A4-1). However, here PLGA-induced CCR gene expression did not cluster or relate closely with ultrapure-LPS induced expression patterns (Fig. A4-1), unlike was previously found for TLRs and integrins. This may be a key difference between PLGA and PAMP stimulation. In particular, CCR7 up-regulation in gene expression is highly associated with DC maturation and contributes to DC committed chemotaxis towards lymph nodes (Sanchez-Sanchez et al. 2006). This was found for DC treated with ultrapure-LPS, as expected, but not found when DCs were treated with PLGA films or agarose films (Fig. A4-1). However, low to mid levels of CCR7 on DCs may play a role in steady-state DC migration to lymph nodes as has been shown in iDCs (Sanchez-Sanchez et al. 2006). It is possible that levels of CCR7 found following PLGA stimulation maintain this DC steady-state migration similar to iDCs. But as was mentioned in CHAPTER 7, RNA gene expression does not always lead to functional protein regulation. Therefore, flow cytometry analysis would be needed to determine CCR7 protein expression on PLGA-treated DCs and functionality in a migration assay in response to CCL19 chemokine.

APPENDIX 5

A.5 ANTIBODY BLOCKING OF TLR4 ON TLR4-EXPRESSING 293 CELLS

As another method to determine if the response of TLR4-expressing HEK293 cells to both LPS and biomaterials was in fact TLR4-dependent (CHAPTER 5), antibody-blocking techniques against TLR4 were utilized. Briefly, TLR4-expressing HEK293 cells were pre-incubated with a-TLR4 blocking antibody (HTA-125, eBioscience, San Diego, CA) or isotype controls for 1hr at a concentration of 20μg/mL. TLR4-expressing HEK293 cells were plated in 100mm TCPS dishes and left untreated or treated with LPS (1ug/mL) as described in CHAPTER 5. The ability for anti-TLR4 to inhibit LPS-induced activation was examined via ability to inhibit L-8 secretion. LPS stimulated the production of IL-8 in TLR4-expressing HEK293 cells; however, the amount of IL-8 secreted by these cells with an a-TLR4 or isotype pre-treatment was the same as with no antibody (Fig. A5-1). Therefore, a-TLR4 blocking techniques using this particular mAb, were ineffective in preventing LPS/TLR4 interactions in TLR4-expressing HEK293 cells, but it has been shown to be effective in primary cells (Termeer et al. 2002; Mirlashari and Lyberg 2003). This difference may be due to TLR4 over-expression which may be above physiological relevance, and therefore difficult to completely inhibit using antibodies. Others have had success in blocking TLR4-expressing 293 cells response to LPS using a lipid A-like compound, E5531 (Uehori et al. 2003) which may prevent TLR4/LPS interactions by binding with higher affinity than a-TLR4. Furthermore, there has been success with antibody blocking to prevent TLR4-induced IL-8 secretion in TLR4-expressing HEK293 cells with a non-LPS ligand (neutrophil elastase) (Devaney et al. 2003). Here, it was possible that impurities in LPS, which are known to include other TLR-stimulatory ligands such as lipoproteins (Hirschfeld et al. 2000), were inducing IL-8 secretion in a TLR4-independent manner. As another explanation, the over-expression of TLR4 in

HEK293 cells may make it unlikely to inhibit their response to LPS as physiological TLR4/LPS interaction on monocytes can easily be prevented using antibody blocking (Flo et al. 2002).

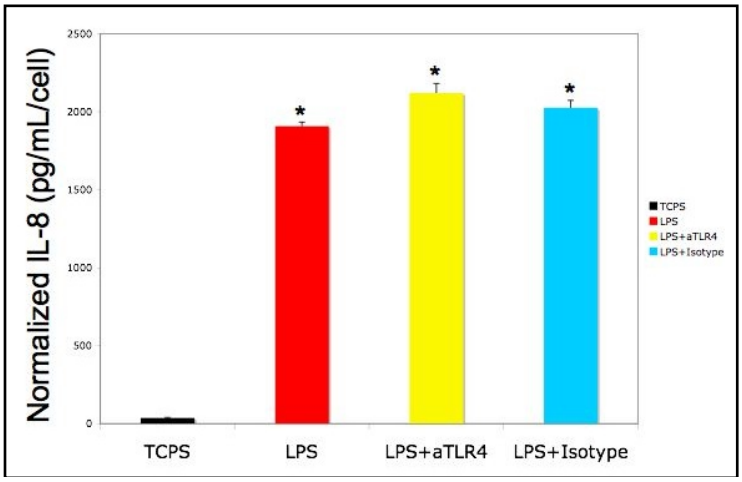

Figure A5-1: IL-8 Secretion of LPS-stimulated TLR4-expressing 293 cells in the presence of a-TLR4. Concentrations were determined from supernatants as measured by ELISA (and normalized to collected cell counts) of TLR4-expressing 293 cells left untreated (TCPS), treated with 1 μg/mL LPS, pre-treated with TLR4-antibody (aTLR4) or isotype for 1 hr followed by treatment with LPS. TLR4-antibody did not block LPS-induced IL-8 secretion. * indicates $p < 0.05$ compared to TCPS (ANOVA), n=1.

APPENDIX 6

A.6 IMMUNOFLUORESCENCE OF ADHERENT HUMAN DCS ON PLGA

Adherent DCs on PLGA films were further imaged using immunofluorescence and analyzed by either standard fluroscent microscopy or confocal microscopy as described in detail in CHAPTER 7. To determine whether B cells, the contaminating cell population in the human-monocyte derived DC culture, adhere to PLGA, the adherent cells on PLGA were stained with CD19-FITC/CD86-PE and DAPI nuclei stain. As shown below, there was no detection of B cells ($CD19^-$) among the adherent cell population (Fig. A6-1) at 24hr following culture. This was in contrast to detection of DC-$SIGN^+$ DCs which adhered readily to PLGA at 24hr (Fig. 7-1). Therefore, it is believed that the primary cellular/biomaterial interaction in the human DC culture occurs between DCs and the biomaterial, as B cells do not tend to directly adhere to the PLGA surface.

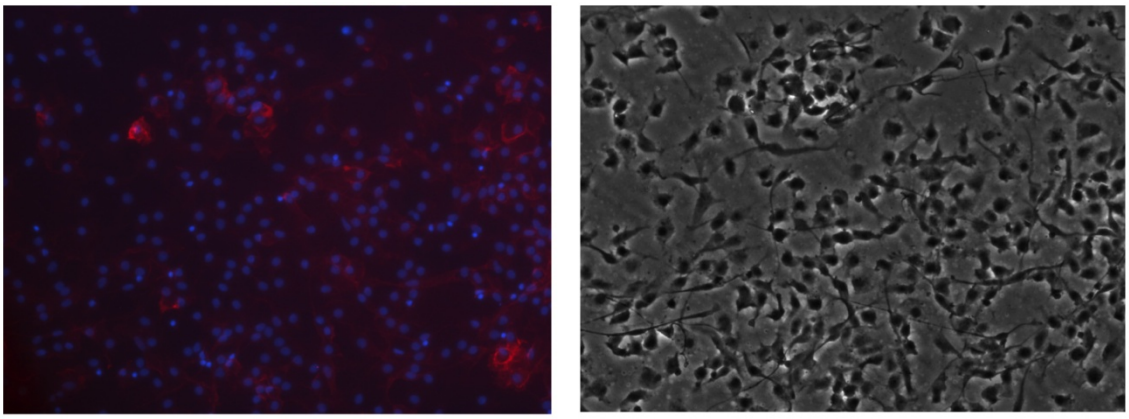

Figure A6-1: CD19/CD86 staining of PLGA film adherent cells. Cells were imaged with CD19-FITC (green), CD86-PE (red) or DAPI nuclei stain (blue) and imaged according to method described in detail in CHAPTER 7, 10X. Fluorescent image (left) with simultaneous bright field (right).

Further evidence of β_2/F-actin podosome co-localization seen in CHAPTER 7 is shown in Fig. A6-2 below.

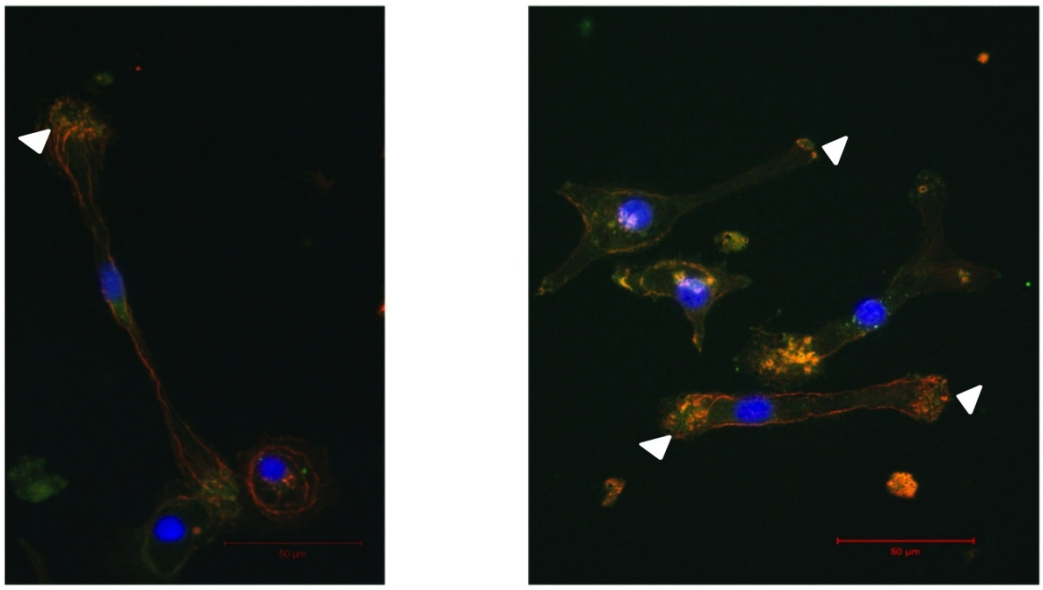

Figure A6-2: β_2 integrin expression on DCs adhering to PLGA films. DCs were allowed to adhere to PLGA films for 1.5h and subsequent were fixed, permeabilized and stained with anti-β_2-FITC in combination with phalloidin-TRITC (red) and nuclei (green). Both images are 40X. White arrowheads indicate areas of co-localization.

Lastly, lack of β_1/F-actin podosome co-localization seen CHAPTER 7, is further visualized below in Fig. A6-3.

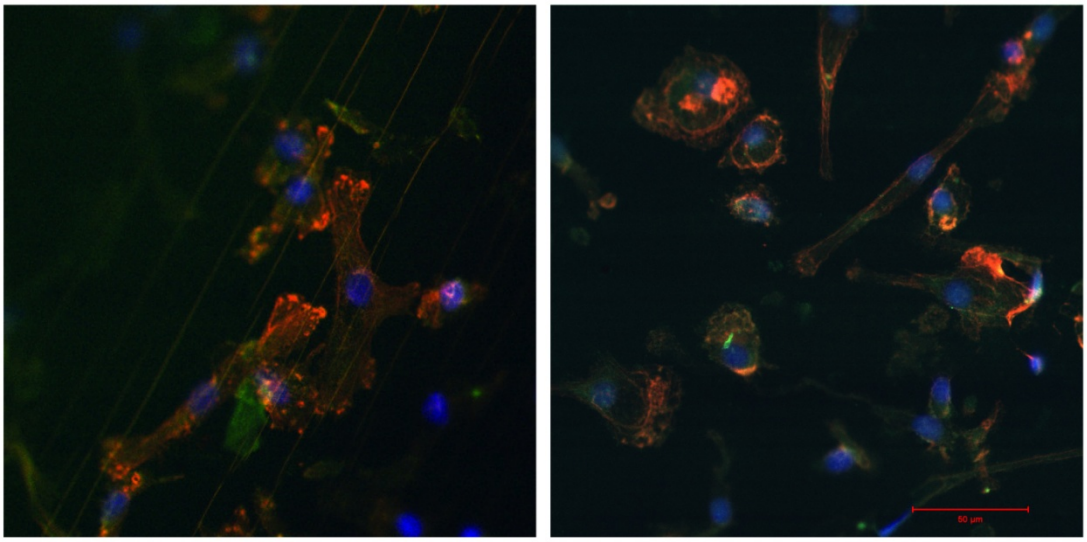

Figure A6-3: β_1 integrin expression on DCs adhering to PLGA films. DCs were allowed to adhere to PLGA films for 1.5h and subsequent were fixed, permeabilized and stained with anti-β_1-FITC in combination with phalloidin-TRITC (red) and nuclei (green). Both images are 40X.

APPENDIX 7

A.7 PROBING BIOMATERIAL 'DANGER SIGNAL'/RECEPTOR INTERACTIONS USING CELL-FREE SYSTEMS

As an independent method to determine the presence of biomaterial 'danger signal'/ receptor interactions, a cell-free system was attempted which utilized soluble recombinant cellular receptors to recognize biomaterial-adsorbed proteins. Specific receptors that recognized adsorbed molecules could then be cross-linked to the surface, and their presence determined using ELISA-based techniques. This system was based off a previously published method, which was used to determine integrin-specific binding to particular pre-adsorbed ligands (Eble et al. 1998). Two receptors of interest, which were shown to be relevant in DC-biomaterial interactions (TLR4, CHAPTER 4/5; and an integrin family member, $\alpha_M\beta_2$, CHAPTER 7), were chosen for examination. Though $\alpha_M\beta_2$ was not directly shown to be involved in DC recognition of biomaterials (CHAPTER 7), the biology of integrin binding indicates the need for both an α and β subunit. Biomaterial recognition by monocytes has been determined to be mediated, in part, by $\alpha_M\beta_2$ (McNally and Anderson 1994); therefore, it is relevant integrin pair for investigation.

Ligands for recombinant TLR4/MD2 (R&D Systems) or $\alpha_M\beta_2$ (R&D Systems), (LPS (Sigma, 20µg/mL) or fibrinogen (Sigma, 100µg/mL), respectively) as well as PBS controls were pre-incubated across wells of 96-well plate (100µL per well) overnight at 4^oC with plates sealed. For TLR4/LPS trials, negative control ligands were also examined using BSA (1%w/v) pre-coating. The next day, wells were washed 3 times in PBS (without Mg/Mn, Invitrogen) and sites were blocked using with 5% non-fat dry milk in PBS/Tween 20 (0.1%) (Sigma) for 1.5hr. Wells were immediately washed with PBS three times. Soluble TLR4 at increasing concentrations (0-100nM, 100µL) was added to wells, in the presence of $MgCl_2$ (2mM) and $MnCl_2$ (1mM), pre-coated with LPS, BSA or

PBS or to LPS-coated wells in the presence of 1mM EDTA to chelate divalent ions and determine integrin-specific binding for 2hr, Soluble $\alpha_M\beta_2$ (20nM concentration, appropriately determined through titration experiment) was added to wells coated with or without fibrinogen and with or without the presence of Mn^{++}, all diluted in 1% BSA/PBS for 2hr. Wells were washed with 25mM HEPES (Sigma) with Mg/Mn three times. Bound receptors were fixed with 2.5% glutaraldehyde (Polysciences Inc) for 10min and further rinsed three times in HEPES. Polyclonal antibodies against hTLR4 and hβ_2 (both from R&D Systems) were added at 1:300 dilution in 1% BSA for 90 min at room temperature. Wells were then washed three times with PBS/Mg (2mM) buffer. Appropriate secondary antibodies conjugated to alkaline phosphatase were diluted (1:300) in 1% BSA and added to wells for 1hr at room temperature. After a final rinsing procedure, signal was processed using pNpp substrate (Sigma) addition and read at 405nm.

For $\alpha_M\beta_2$, specific detection of the bound integrin was determined by testing all combinations of receptor (with or without Mn), primary and secondary additions following the pre-incubation of fibrinogen or PBS controls (Fig. A7-1). It was found that $\alpha_M\beta_2$ was specifically being detected by this ELISA-based technique; however, $\alpha_M\beta_2$-fibrinogen binding (a known ligand pair) gave a lower signal in the presence of fibrinogen than without its pre-coating (Fig. A7-1). Also, Mn presence was not found to affect the binding of $\alpha_M\beta_2$ to fibrinogen (Fig. A7-1).

The detection of bound recombinant TLR4 on LPS, BSA or PBS pre-coated surfaces using increasing concentrations of TLR4 did not result in significant increase in signal (Fig. A7-2). The presence of EDTA, which chelate ions necessary for integrin binding had no significant effect TLR4 binding/detection (Fig. A7-2). LPS coated surfaces yielded similar signal to uncoated surfaces as well as surfaces coated with BSA

indicating that only non-specific adsorption of TLR4 to surface may be being detected even in the presence of its known ligand (Fig. A7-2).

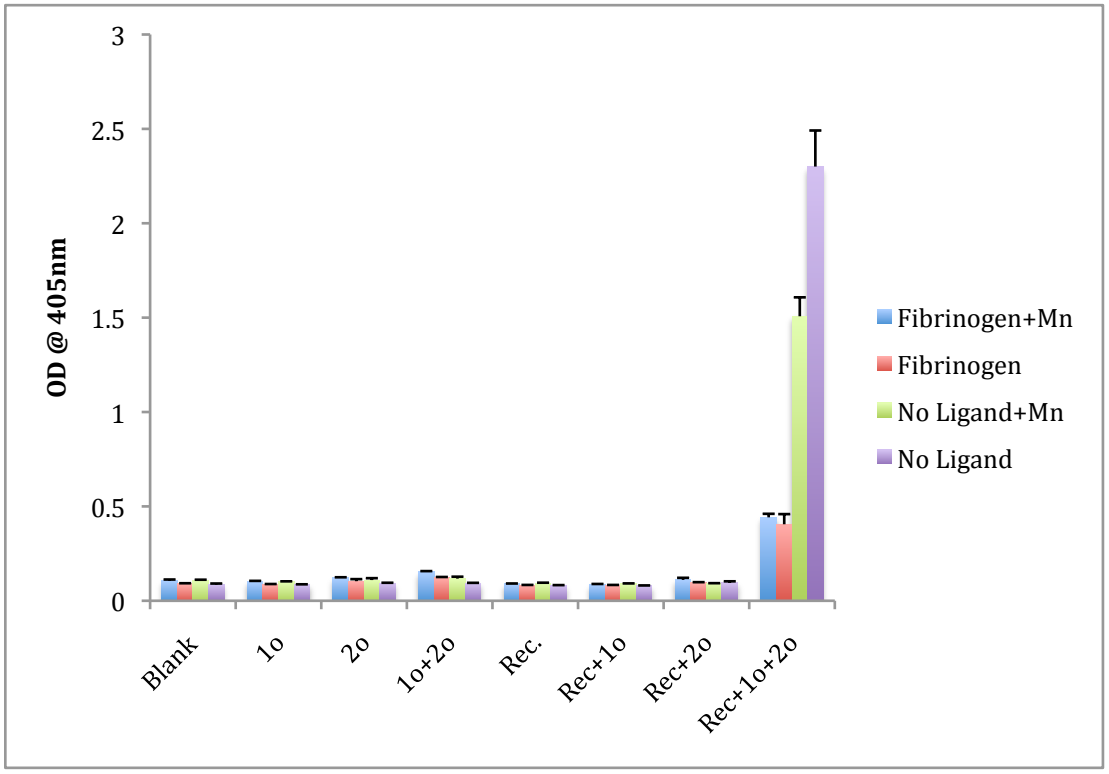

Figure A7-1: Specific detection of $\alpha_M\beta_2$ binding on fibrinogen or PBS (No Ligand) surface. Plates were coated with or without fibrinogen and $\alpha_M\beta_2$ was added to the wells with or with the presence of Mn. Lastly, specific detection of $\alpha_M\beta_2$ (Rec.) was determined by analyzing wells with primary (1^o) or secondary (2^o) alone or in combination to determine specific. The receptor (Rec) in the presence of both primary and secondary only resulted in significant signal. Bars indicate mean+s.d. (in triplicate).

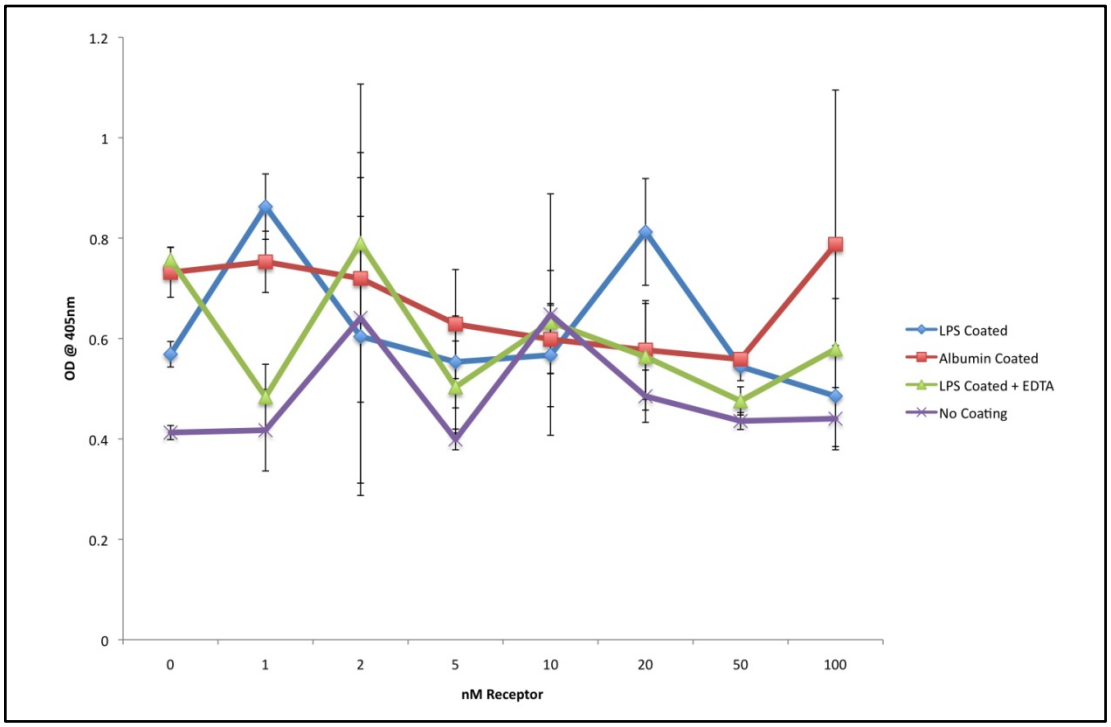

Figure A7-2: Detection of increasing concentrations of recombinant TLR4 binding to LPS, BSA (Albumin) or No Coating Surfaces. Plates were coated with LPS (blue), BSA (red) or PBS (purple) and recombinant TLR4 was added to the wells in the presence of Mn/Mg or in the presence of 1mM EDTA (LPS coated + EDTA, green). Dots indicate mean±s.d.

Using this cell-free ELISA-based system, the ability to detect recombinant receptors was possible as shown in Fig. A7-1. However, for either receptor investigated (TLR4, or $\alpha_M\beta_2$), pre-adsorbed known ligand did not lead to increases in receptor detection. $\alpha_M\beta_2$ is known to bind to fibrinogen (Tang et al. 1996) and TLR4 is well known to recognize LPS. However, $\alpha_M\beta_2$ was detected at a higher level on uncoated surfaces than on surfaces coated with fibrinogen (Fig. A7-1), and TLR4 was found at similar levels regardless of surface coating (Fig. A7-2). Therefore, the detection of either $\alpha_M\beta_2$ or TLR4 is not dependent on specific substrate recognition as would be needed to verify role in biomaterial recognition but instead is based on non-specific protein adsorption.

For this reason, the use of this cell-free system for the detection of specific receptor-ligand interactions on biomaterials did not seem feasible.

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